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SINGLE ENZYME MOLECULE ANALYSIS BY CAPILLARY ELECTROPHORESIS

by

Robert Polakowski

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Chemistry

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SINGLE ENZYME MOLECULE ANALYSIS BY CAPILLARY ELECTROPHORESIS

Abstract

The study of single molecules (SMs) is a field which, although not new, has only begun to show its potential. With the development of ever more sensitive instrumentation the wonderful world of chemistry can now be probed on an individual molecule basis. The findings are sometimes predictable, sometimes unexpected or confusing, but invariably exciting.

One of the disciplines which have embraced this approach is enzymology. The complexity of enzymes means that SM approach is possibly the only way to fully understand their behaviour. Using capillary electrophoresis with laser-induced fluorescence as a tool allows for some interesting insights into the behaviour of individual enzyme molecules.

Alkaline phosphatases (AP) from various sources were studied in the course of this research on an indvidual molecule basis. Initially, the relationship between enzyme structure and its activity was explored. Calf intestinal AP molecules were previously shown to very heterogeneous with respect to their activities and a number of possible reasons were postulated. AP of *E. coli* was then investigated. The initial heterogeneity in activities of SMs of a commercial preparation of this enzyme was shown to be the result of extensive enzyme degradation. When three isoforms of this enzyme were purified to high homogeneity, activities of SMs within each of the isoforms were nearly identical. There was also very little observable difference between the isoforms - corresponding to very small differences in their structures.

Thermal denaturation of *E. coli* AP was studied on a SM basis and was shown to be a catastrophic process, whereby molecules lost their activities abruptly upon heating.

The kinetics of single calf intestinal AP molecules were investigated. The molecules were immobilized within a capillary and repeatadely incubated in various

concentrations of substrate. The results again showed the great heterogeneity of SMs of this particular isoenzyme. The average reaction velocity and Michaelis constant of all individual molecules agreed well with those found for the bulk sample. This showed the SM approach to be valid and superior to conventional bulk methods.

Finally, other AP isoenzymes were briefly looked at and some improvements were made to the instrumental set-up.

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Didn't I say I couldn't have done it alone...?

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List of Abbreviations

2A2M1P	- 2-amino-2-methyl-1-propanol
2D-PAGE	- two-dimensional polyacrylamide gel electrophoresis
6HFG	- 6-hydroxyfluoran-β-D-galactopyranoside
Ala	- alanine
AP	- alkaline phosphatase
APS	- ammonium persulfate
Arg	- arginine
Asn	- asparagine
Asp	- aspartic acid
ATP	- adenine triphosphate
ATPase	- adenine triphosphatase
AttoFluor	- 2'-[2-benzothiazoyl]-6'-hydroxybenzothiazole (also see **BBT**, and **FE**)
AttoPhos™	- 2'-[2-benzothiazoyl]-6'-hydroxybenzothiazole phosphate (also see **BBTP**)
B	- base
BBT	- 2'-[2-benzothiazoyl]-6'-hydroxybenzothiazole (also see **AttoFluor**, and **FE**)
BBTP	- 2'-[2-benzothiazoyl]-6'-hydroxybenzothiazole phosphate (also see **AttoPhos™**)
BH^+	- protonated base
BK-AP	- bovine kidney alkaline phosphatase
BSA	- bovine serum albumin
CE	- capillary electrophoresis
CI-AP	- calf intestinal alkaline phosphatase

CP - central peak

CS - conformational substate(s)

CW - continuous wave

δ - thickness of the double layer

DEA - diethanolamine

DEAE - diethylaminoethyl

ddH_2O - distilled, deionized water

DMSO - dimethyl sulfoxide

DNA - deoxyribonucleic acid

DTT - dithiothreitol

e - charge per unit surface area

E - enzyme or applied electric field, as apparent from context

ε - dielectric constant

E. coli - *Escherichia coli*

EAE - ethylaminoethanol

EC-AP - *Escherichia coli* alkaline phosphatase

EDTA - ethylenediaminetetraacetic acid

ELISA - enzyme-linked immunosorbent assay

EOF - electroosmotic flow

ER - endoplasmic reticulum

ES - enzyme-substrate complex

EtOH - ethanol

EU - enzymatic units

FE - fluorescent emitter (also see **AttoFluor** and **BBT**)

FIA - flow injection analysis

FITC - fluorescein isothiocyanate

FRET - fluorescence resonance energy transfer

GMBS - N-succinimidyl-4-maleimidobutyrate

GPI - glycosylphosphatidylinositol

HAc - acetic acid

HP-AP - human placental alkaline phosphatase

i.d. - internal diameter

IEF - isoelectric focusing

IEPBR - immobilized enzyme packed-bed reactor

IgG - immunoglobulin G

Ile - isoleucine

k - Boltzmann distribution constant

kDa - kilo-Daltons

kJ - kilo-Joules

$\mathbf{K_m}$ - Michaelis constant

$\mathbf{k_x}$ - rate constant for step x (in an enzymatic reaction pathway)

kat - katal (unit of enzymatic activity)

LDH-1 - lactate dehydrogenase 1

LIF - laser-induced fluorescence

M - molar (moles per liter)

μ_{EP} - electrophoretic mobility

MALDI-MS - matrix assisted laser desorption ionization - mass spectrometry

MeOH - methanol

mRNA - messenger ribonucleic acid

MTS - mercaptopropyl trimethoxysilane

mW - milli-Watts

MW - molecular weight

MWCO - molecular weight cut-off

η - viscosity

NAD^+	- nicotinamide adenine dinucleotide
N.D.	- neutral density
o.d.	- outer diameter
P	- product (of enzymatic reaction)
P1-P6	- peaks 1 through 6
PA	- polyacrylamide
PAGE	- polyacrylamide gel electrophoresis
pI	- isoelectric point
pK_a	- neagtive logarithm of acid dissociation constant
PK-AP	- porcine kidney alkaline phosphatase
PMT	- photomultiplier tube
pNPP	- p-nitrophenyl phosphate
q	- electrical charge
r	- radius
RFU	- relative fluorescence units
RNA	- ribonucleic acid
RTD	- resistive thermal device
s	- seconds
S	- substrate
S-AP	- shrimp alkaline phosphatase
SDS	- sodium dodecyl sulfate
SDS-PAGE	- sodium dodecyl sulfate - polyacrylamide gel electrophoresis
Ser	- serine
SM	- single molecule
t	- time
T	- temperature
TCA	- trichloroacetic acid

TEMED	- N,N,N',N' - tetramethylethylenediamine
Thr	- threonine
TMR	- tetramethyl rhodamine
Tris	- Tris (hydroxymethyl) aminomethane
TRTC	- tetramethylrhodamine thiocarbamyl
U	- (enzymatic) units (also see **EU**)
USDOE	- United States Department of Energy
υ	- reaction velocity
υ_{EP}	- electrophoretic velocity
V_{max}	- maximum reaction velocity
V_o	- reaction velocity
ψ	- charge on the enzyme
ζ	- zeta potential
ze	- charge on substrate

1

Introduction

1.1 ENZYMOLOGY

Enzymology is the study of enzymes - catalytically active proteins (with a notable exception of ribozymes, enzymatically active RNA molecules) that are responsible for all life on this planet. The catalytic power of the enzymes is astounding - they are capable of accelerating reaction rates by up to 10^{14} times while working optimally, for most part, under ambient conditions. Without enzymes few biologically important reactions would proceed at appreciable rates. Most enzymes are also highly specific, both in the nature of their substrates and in the reactions they catalyze. The low specificity enzymes are those exhibiting bond specificity, that is a preference for a particular bond without much constraint on the rest of the substrate. Alkaline phosphatase, the molecule studied throughout this thesis, is an example of a low specificity enzyme. It will cleave any phosphoester bond regardless of the substrate. The classification of enzymes is the domain of the Enzyme Commission which categorizes all enzymes into 6 types, depending on the reaction they catalyze. These types are:

1) oxidoreductases - catalyzing oxidation-reduction reactions,

2) transferases - catalyzing group transfer reactions,

3) hydrolases - catalyzing hydrolytic cleavage reactions,

4) lyases - catalyzing elimination reactions with a formation of a double bond,

5) isomerases - catalyzing isomerization reactions,

6) ligases (or synthetases) - catalyzing synthesis reactions and utilizing some form of energy source.

Alkaline phosphatase is a type 3 enzyme.

Like all proteins enzymes are composed of long chains of amino acids joined together via a peptide bond. The sequence of amino acid in a protein is specified by the DNA sequence of a gene responsible for this protein in an organism. Proteins are three dimensional structures, having three to four levels of structural complexity.

The primary protein structure refers to the linear arrangement of the amino acids and also includes all covalent linkages within the polypeptide chain. The polypeptide chain is built up in the process of translation, when the information contained within an mRNA molecule is translated into the amino acid sequence at the ribosomes inside cells. There are 20 amino acids coded for by a genome.

The secondary structure involves the arrangement of segments of this chain into regular structures such as α-helix, β-sheet, and β-barrel. This arrangement is specified by the amino acid sequence of an enzyme.

The tertiary structure refers to further folding of 2° structures into a more compact form. Again, the three dimensional structure under physiological conditions is already specified by the amino acid sequence. Disulfide bridges, which can form between cysteine molecules, are sometimes seen to form once the tertiary structure is achieved. Their purpose is the further stabilization of the 3° structure.

The 4° structure exists for proteins composed of more than one subunit. The subunits can be held together by a variety of interactions including electrostatic, van der Waals, and hydrophobic interactions, H-bonding and sometimes disulfide bridges.

The driving force behind the folding of a polypeptide chain into a three dimensional structure is a combination of two factors - the hydrophobic effect and the existence of non-covalent interactions between the amino acids of the polypeptide chain. In a physiological environment, surrounded by water molecules, amino acids with hydrophobic side chains will tend to arrange themselves so as to exclude the water from their immediate environment. This will involve certain ways of folding which will bring together amino acids which may be quite far apart in the linear arrangement. When these amino acids come together, other types of forces, such as electrostatic interactions, hydrogen bonding, and van der Waals forces come into play, further folding and stabilizing the polypeptide chain. In the process, the molecule can go through a number of conformational substates (CS), separated from each other by

potential energy barriers. Some of these substates are more favourable than others and into these the protein may settle for a while before switching to another CS. This view of protein folding is known as the energy landscape model and is well described in a number of papers published by Frauenfelder[1-3]. The process is clearly dynamic; the protein constantly moves between the CSs, so that the overall structure is an average of the many conformations explored by the molecule. The time scales for these fluctuations appear to be small, on the order of 10^{-14} to 10^{-8} for different types of fluctuations[4]. The influence that this CS switching has on the function of proteins is being currently studied and the single molecule approach may prove to be very useful.

For many enzymes folding alone does not end in a biologically active molecule. In addition to amino acids, enzymes in their active forms may also contain other non-protein components known as cofactors or, in case of covalently bound cofactors, as prosthetic groups. Two classes of these are metal ions and organic molecules such as biotin, flavin nucleotides, or lipoic acid. Enzymes containing cofactors are termed haloenzymes; those which had their cofactors removed are also known as apoenzymes. Enzymes can also be post-translationally modified through the action of proteases (enzymes which cleave peptide bonds), or through glycosylation (addition of oligosaccharides), phosphorylation (addition of phosphate groups), or acylation (addition of lipid moieties).

Separate from the issue of CSs is the existence of isozymes, or isoenzymes. The definition of an isoenzyme is a shaky one. For some, isoenzymes are different forms of an enzyme that arise from genetically determined differences in the amino acid sequence. A different definition proposes that the term should be applied to all molecular forms of an enzyme which can be resolved by any of the existing separation techniques. Isoelectric focusing (IEF) is often taken as the most powerful technique for the separation of isozymes. According to the first definition, among multiple forms of alkaline phosphatase, isozymes would be those phosphatases whose production is

controlled by different genes, i.e. bovine intestinal alkaline phosphatase, human placental alkaline phosphatase, *E. coli* alkaline phosphatase, etc. Yet within each of these groups multiple isoforms can still be resolved using chromatography or electrophoresis (especially IEF). According to the second definition, these are also termed isozymes. The second definition will be accepted in this thesis with a small distinction. When talking about multiple forms of an alkaline phosphatase from a single source (i.e. a product of a single gene) the terms isoform will be used. The term isozyme (or isoenzyme) will be used to describe AP variants coded by separate genes (i.e. coming from different tissues). And so I will speak of *E. coli* AP isoforms, calf intestinal AP isoforms, or human placental AP isoforms, but of *E. coli* vs. calf intestinal isoenzymes. The reader is asked to forgive any confusion this may cause.

As catalysts enzymes can be said to have certain activities. The activity of an enzyme is the rate with which it catalyzes a reaction. Activity of enzymes can be expressed in various forms, the most popular being in units of μmol substrate consumed or product produced per minute (μmol/min.). Other possibilities include the enzyme unit (U or EU, equal to μmol/min.), the katal (kat, where 1 U = 16.67 kat), or M/s (where the conversion will depend on the sample volume). The activities of enzymes are heavily dependent on a number of factors such as temperature, pH, solvent type, presence of cofactors, presence of certain inhibitors.

The understanding of an enzyme's mechanism of action is achieved through the study of its kinetics. For one-substrate reactions, such as those catalyzed by hydrolyses, the simplest way to describe the reaction is given by the equation:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

Equation 1.1

where E is the enzyme, S - the substrate, ES - the enzyme-substrate complex, P - the product, k_1 - the rate constant for the formation of the ES complex, k_{-1} - the rate constant for the reverse reaction, and k_2 - the rate constant for the dissociation of the ES

complex to form the product. Using either the equilibrium assumption or the steady-state assumption, the kinetics of the above scheme can be described by the equation:

$$v = \frac{V_{max}[S]}{K_m + [S]} \qquad \textbf{Equation 1.2}$$

where v is the reaction velocity, V_{max} is the maximum reaction velocity, $[S]$ is the substrate concentration, and K_m is the Michaelis constant, equal to the concentration of substrate when the reaction velocity is half maximal. V_{max} can also be expressed as the product of initial enzyme concentration and ES complex dissociation constant (k_2), also known as the turnover number, and is then equal to $k_2[E]_0$. The above equation is referred to as the Michaelis-Menten equation after a pair of scientists who were among the first to describe enzyme kinetics in these terms[5]. More correctly, it should be called the Henri-Michaelis-Menten equation, to give credit to Victor Henri, the first person to apply systematic mathematical treatment to enzymatic reactions[6]. Kinetic data is obtained by measuring v at different substrate concentrations and is then analyzed by plotting reaction velocity against substrate concentration. In order for the above assumptions to hold true, reactions must be carried out under substrate saturation conditions, so that the overall rate of the reaction will depend on the rate of ES complex dissociation to give the product. A computer algorithm based on **Equation 1.2** can be used to extract V_{max} and K_m, the two constants used to characterize enzymes.

Alkaline phosphatase follows the Michaelis-Menten kinetics. The precise reaction mechanism is still not fully understood and so it will not be presented here to avoid any confusion. **Equation 1.2** is sufficient to describe the mechanism for the purpose of this work. It will be encountered again in **Chapter 4**.

The preceding section was just the briefest possible introduction to some simple enzymology concepts that will be encountered throughout the thesis. The field itself is enormous and for further reading one should choose one of the many texts providing a thorough review[7-10].

1.2 SINGLE MOLECULE ENZYMOLOGY

Whereas enzymology has been a part of sciences for quite some time now, including the long period when the nature of enzymes was not yet understood, single molecule enzymology is a comparatively new field. Although one does not need to understand what enzymes are to recognize their action and to appreciate their potential, studying single enzymes requires a firm grasp of the concepts of enzymology, chemistry, as well as of the various techniques that will allow the capture and observations of these small engines that make all life possible. The introduction to this field will contain some history, the major players in the area today, and some concepts specific to the study of single molecules.

1.2.1 Early Work

Single molecule enzymology has its beginnings in the early 1960s, in the work of Boris Rotman, then at the Department of Genetics of the Stanford University School of Medicine[11]. He used a novel fluorogenic substrate, 6-hydroxyfluoran-β-D-galactopyranoside (6HFG), to measure the molecular weight and turnover number of individual molecules of β-D-galactosidase. Very dilute solution of stabilized enzyme was sprayed onto glass coverslips, the microscopic droplets (14-15 μm in diameter) were coated with silicone oil, and the fluorescence readings were taken using a Zeiss optical microscope after 15 hrs of incubation. Rotman found that the molecular weight (MW) and turnover number agreed well with those obtained in conventional measurements. He was also the first to propose the use of such techniques in determining the homogeneity of an enzyme species with respect to activity. He found that genetically altered β-D-galactosidase had a different activity from that of the normal enzyme. Unfortunately, the extensive sample preparation and very long incubation times made Rotman's approach too cumbersome; a long period of inactivity in the field of single molecule detection followed.

In the mid-1970s Theodore Hirschfeld, then at Block Engineering, Inc., used fluoresceine isothiocyanate (FITC) labeling to detect single molecules of an antibody, γ globulin[12,13]. He attached between 80 and 100 molecules of FITC to a polyethyleneimine (MW 20,000) molecule which served as a backbone to which both the dye and the antibody were bound. Using an optical microscope he then observed the antibody molecules as they passed through an illuminated region. This approach to single molecule detection also proved to be too difficult and time consuming and was not repeated.

A great deal of progress in detecting single molecules in flowing solutions was made throughout the 1980s by Richard Keller's group at Los Alamos. Keller's work allowed the development of sensitive laser-induced fluorescence (LIF) techniques for the detection of molecules passing through a detection volume. This was a significant improvement on the earlier work where the signal from a stationary molecule was integrated over the long incubation times employed. In the early 80's the Keller group included Norman Dovichi; during that time instrumental improvements were explored which would allow the detection of single molecules of a fluorescent dye, rhodamine 6G, using a flow cytometer system[14-16]. These efforts culminated in the successful laser-induced fluorescence detection of single molecules of phycoerythrin in hydrodynamically focused flows[17] and later in the LIF detection of single molecules of rhodamine 6G in ethanolic solutions[18]. The Keller group continued working with detection of single dye molecules throughout the 1990s publishing a number of papers dealing with the detection, fluorescent lifetimes, optical trapping, collection efficiency, and other aspects of single molecules in flowing streams[19-27]. Although Keller's work never dealt specifically with enzymes, his contribution to the field is undoubtedly significant, not the least because his work sparked the interest of my own supervisor, N. J. Dovichi, in this area that eventually led to this thesis.

1.2.2 To wards Single Molecule Enzymology: Work on Improving Detection Limits and on Single Enzyme Molecules in the Dovichi Group

The work of Norman Dovichi in SM enzymology continued at the University of Alberta. The initial research was concerned with progressive lowering of detection limits for various dyes, and dye-labeled compounds, using the in-house built laser-induced fluorescence capillary electrophoresis instruments utilizing a sheath-flow cuvette detection chamber (which will be described in **Section 1.3.3**). Detection limits in the low zeptomole (1 zeptomole = 1×10^{-21} moles = 600 molecules) to low yoctomole (1 yoctomole = 1×10^{-24} moles = 0.6 molecule) range were routinely achieved for fluorescein isothiocyanate (FITC)-labeled and tatramethylrhodamine thiocarbamyl (TRTC)-labeled amino acids[28-30], intercalating dye-labeled DNA[31,32], dye molecules[33,34], dye-labeled monosaccharides[35], and dye-labeled mono- and oligo-saccharide products of enzymatic reactions[36,37]. The issue of molecular shot noise as a fundamental limit in chemical analysis was dealt with in a paper published by Chen and Dovichi in 1996[38]. Progress was also being made in the fluorescence-based enzymatic assays: two papers published in 1995 and 1996 by Craig *et al.* describe the detection of 1.6 molecules of β-galactosidase and 9 molecules of alkaline phosphatase[39,40]. The ultimate goal of single enzyme molecule detection was finally achieved by Craig *et al.* for molecules of calf intestinal and *E. coli* alkaline phosphatases[41-43], and later for β-galactosidase of *E. coli*[44]. The alkaline phosphatase work laid the foundation for the work described in this thesis and it will be briefly described here and referred to throughout the thesis.

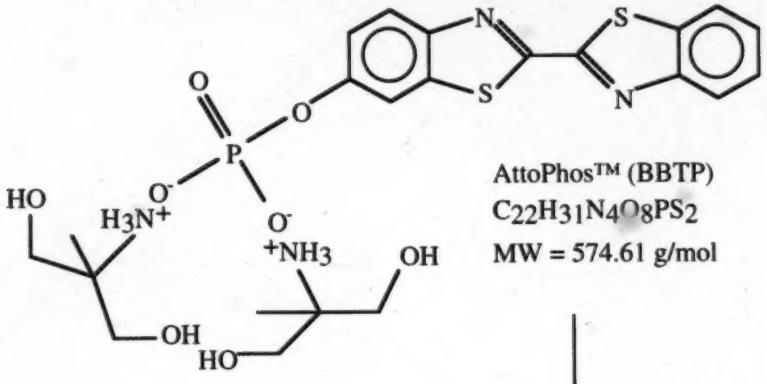
In order to detect single molecules of AP Craig *et al.* utilized a then-novel fluorogenic substrate for this enzyme, 2'-[2-benzthiazoyl]-6'-hydroxy-benzthiazole phosphate, sold as a Bis-[2-amino-2-methyl-1,3-propanediol] salt ($C_{22}H_{31}N_4O_8PS_2$, MW 574.61 g/mol) under a trade name AttoPhos™ (JBL Scientific, San Luis Obispo,

CA; alternatively known as BBTP). AttoPhos™ has a pH optimum between 9.0 and 10.3 and the K_m for it with AP is 0.03 mM in 2.4 M diethanolamine (DEA), 0.057 mM $MgCl_2$, pH 10.0 buffer. The K_m for AP with AttoPhos™ in the 100 mM borate buffer, pH 9.5, which was extensively used in D. Craig's CE single molecule assays, was determined in a bulk assay to be 0.15 mM. This weakly fluorescent molecule is converted in the course of the enzymatic reaction to a highly fluorescent dephosphorylated counterpart, 2'-[2-benzthiazoyl]-6'-hydroxy-benzthiazole ($C_{14}H_8N_2OS_2$, MW 284.35 g/mol), known as Fluorescent Emitter (FE; alternatively AttoFluor, or BBT). Both structures are shown in **Figure 1.1**. Fluorescent Emitter is excited in an optimal wavelength range of 430-440 nm and it has an emission maximum at 550-560 nm, giving it a large Stokes' shift of 120 nm, and so making it superbly suitable for laser-induced fluorescence detection. Although AttoPhos™ has a similar excitation maximum, its Stokes' shift is only 20 nm, and the intensity of its emission at the FE's emission maximum is only 2-4 % of the FE's intensity. Nevertheless, in an enzyme assay, where the amount of substrate far exceeds the amount of product, AttoPhos™ may cause a large increase in background, sufficient to swamp out the signal from FE. Separation of the two compounds is then necessary.

Craig *et al.* used capillary electrophoresis to achieve the separation of the two compounds. The details of the experiments were published previously[41-43], and the instrument will be described in **Section 1.3.3**. Briefly, a very dilute solution of the enzyme ($\approx 1 \times 10^{-15}$ M) in 90 mM borate/10% DMSO buffer, pH 9.5, was injected electrophoretically onto a fused silica capillary (10 μm i.d., 70 cm long) for 3-5 min. at 400 V/cm. After an incubation (14-30 min.) the contents of the capillary were swept past the detector under a 400V/cm field. The resulting electropherograms contained sets of peaks on top of a low plateau, followed by a higher plateau. The first plateau was due to auto-hydrolysis of AttoPhos™ to FE with the peaks representing the FE generated by the individual enzyme molecules. The following plateau was due to

Figure 1.1 Structures of AttoPhos™ and Fluorescent Emitter, the substrate of alkaline phosphatase, and the product of its hydrolysis.

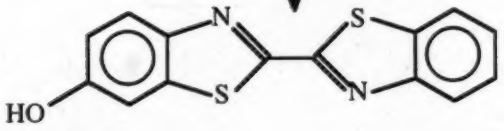
2'-[2-benzothiazoyl]-6'-hydroxybenzothiazole phosphate



AttoPhos™ (BBTP)
$C_{22}H_{31}N_4O_8PS_2$
MW = 574.61 g/mol

+ AP

Fluorescent Emitter
(FE, BBT, AttoFluor)
$C_{14}H_8N_2OS_2$
MW = 284.35 g/mol



2'-[2-benzothiazoyl]-6'-hydroxybenzothiazole

AttoPhos™. A more detailed description of a typical electropherogram will be given in **Section 2.2.3.1**. The assays on single molecules of calf intestinal alkaline phosphatase showed a broad and non-Gaussian distribution of the activities of individual enzyme molecules; the range of activities varied 10-fold. The mean activity was found to be 111 molecules FE/second with the standard deviation of 83 s^{-1}. A number of possibilities for this heterogeneous distribution were postulated. Poor experimental precision, adsorption of the enzyme onto capillary walls, enzyme denaturation during assay, enzyme aggregation were all discarded based on other experiments. Two possibilities remained: post-translational modifications affecting individual molecules to different levels (i.e. the creation of isoforms of the enzyme) or secondary structure fluctuations resulting in some molecules being more or less active than others at any given time.

In the same paper Craig *et al.* described two other aspects of single molecule enzymology. In a related experiment, where a higher dilution of enzyme was employed to achieve the capture of, on average, one molecule of enzyme in the capillary, multiple incubations were performed on the molecule. The molecule was moved into a pool of fresh substrate by means of a short electrophoretic pulse. Temperature was varied for the individual incubations. The peak areas were measured and an Arrhenius plot was constructed to obtain the activation energy for the reaction catalyzed by a single enzyme molecule. There was a 2.3-fold difference between the lowest and highest activation energies. The average energy was found to be 53 kJ/mol with the standard deviation of 16 kJ/mol ($n = 8$). No correlation was found between activation energy and activity.

In another experiment Craig *et al.* studied the death of enzyme molecules as a result of thermal denaturation. There are two possible models for the thermal denaturation of enzymes. The catastrophic model predicts the loss of activity by an enzyme molecule to be total and abrupt, so that the gradual decrease of activity of an enzyme sample upon heating is a result of progressively more molecules losing their

catalytic power while the remaining ones keep on working. The "Cheshire cat" model predicts that individual molecules lose their activities slowly and gradually, and the gradual inactivation displayed by a bulk sample is a simple direct reflection of individual molecules' behaviour. More will be said about thermal denaturation of enzymes in **Section 2.1**; a brief description of the experiment follows. A dilute solution of enzyme was heated at 64°C for 5 min. and immediately diluted another 6 orders of magnitude to quench the denaturation reaction. The final dilution of enzyme was then injected onto the capillary in the presence of substrate and incubated for 15 min. before being swept past the detector. The number of surviving molecules, as compared to an unheated control, was counted and their activity distribution was measured. It was observed that the loss of activity paralleled the loss of active molecules and so it was concluded that the denaturation of calf intestinal alkaline phosphatase was a catastrophic phenomenon.

These experiments paved the way for my investigation into the nature of single enzyme molecules using alkaline phosphatase as a model enzyme. A lot more will be said about the enzyme itself and the thinking behind the experiments in **Chapters 2 - 4** of this thesis.

1.2.3 Single Molecule Enzymology and Single Biomolecule Work in Other Research Groups

The 1990s saw great advances in the area of single molecule studies, including enzymology. Many groups study single enzyme molecules using a variety of approaches and instruments. Publications abound and at least three special issues dedicated to the study of single molecules in general have been published[45-47]. Workshops on the topic (Single Molecule Detection and Ultrasensitive Analysis in the Life Sciences) have been held in Berlin for the past 6 years by PicoQuant. Last year saw possibly the greatest recognition for this field - a Nobel conference on the topic of

Single Molecules in Physics, Chemistry, and Biology was held in Stockholm, Sweden. Finally, last year also saw the inaugural issue of Single Molecules, "an international, interdisciplinary journal covering all aspects of single molecule investigations in chemistry, physics, and life sciences..."[48]. In light of all these advances it would be impossible to offer a thorough review of the subject in this thesis. The contributions of the key players however, will be discussed.

Paralleling our efforts in single enzyme molecule studies is Ed Yeung's group at Ames Laboratory - USDOE and the Department of Chemistry at Iowa State University. In a paper briefly preceding that of Craig *et al.*[41], Xue and Yeung found up to four-fold variations in the activities of individual lactate dehydrogenase - 1 (LDH-1) molecules[49]. The activity of LDH-1 was estimated by measuring fluorescence produced by NADH, a product of enzymatic reaction of LDH-1 with nicotinamide adenine dinucleotide (NAD^+). In an extension of this study Tan and Yeung used simultaneous monitoring of more than 100 microreactors, each containing a single molecule of LDH-1, both in real time and averaged over a 30 minute incubation time[50]. Once again, he found heterogeneity in both the instantaneous rates and time-averaged rates of individual enzyme molecules. His conclusions in both cases suggested an existence of distinct molecular conformations at the 3° level of enzyme structure as the reason for this heterogeneity. We have tested his assumption by subjecting LDH-1 from the same source as published by E. Yeung to isoelectric focusing and found that the enzyme claimed by E. Yeung to be structurally homogeneous was in fact resolvable into a number of bands[51]. It is our opinion that a simpler explanation for this behaviour was offered by Craig *et al.*[41] and later confirmed in the course of this thesis research.

Other areas of Ed Yeung's research in single molecules encompass measurements of single molecule diffusion and photodecomposition in free solutions[52], electrostatic trapping of single protein molecules at liquid-fused silica

surface[53], high-speed and high-throughput single molecule spectroscopy in solutions for identifying single molecules based on their fluorescence emission spectra[54], and single DNA molecule screening[55]. A rather important experiment by E. Yeung concerned a non-enzymatic catalyst, Os(VIII) ion. Tan and Yeung monitored catalytic reaction rates of a number of individual Os(VIII) ions and found the heterogeneity in their reaction rates to be around 15%[50]. They concluded that structurally identical catalysts, such as the simple Os ion, have identical reaction rates provided that the environmental influences are similar molecule-to-molecule.

A different approach to single molecule studies has been taken by X. Sunney Xie's group at the Pacific Northwest Laboratory, Richland, WA and later at Harvard University. His early single molecule work was concerned with probing the dynamics of single molecules of dyes on nano-, milli-, and second time scales[56,57]. In the field of single molecule enzymology, Lu *et al.* have observed enzymatic turnovers in real time of single cholesterol oxidase molecules by monitoring emission from the enzyme's active site[58]. They found slow, stochastic fluctuations in the rate of enzymatic reaction of cholesterol oxidase molecules trapped within an agarose gel matrix as well as heterogeneity in their enzymatic turnovers. They also observed that the turnover of a single molecule was dependent on its previous turnovers through a molecular memory phenomenon. They postulated proteolytic damage of key residues in the enzyme to account for the heterogeneity and slow conformational changes around the active site of the enzyme as a reason for the rate fluctuations and the memory effect. S. Xie's other contribution to the field includes two excellent reviews[59,60].

Real time observation of single molecule events has also been adopted by a number of researchers in Japan. Toshio Yanagida and his group at Osaka University Medical School and associated agencies, have studied ATPase activity of single myosin molecules during their interactions with actin[61,62]. Their studies were also done in real time and the findings contradict the widely accepted view of ATPase activity of

actin-myosin complexes. Instead of actin-myosin contraction immediately following the hydrolysis of ATP, they found the complex to have a memory effect, by which the energy released upon ATP hydrolysis is stored in the myosin molecule for up to several hundred of milliseconds. In a different study Vale *et al.* directly observed, by means of a fluorescent label attached to kinesin, the movement of this motor protein along microtubules[63]. Finally, in a paper published by Ishii *et al.*, fluorescence resonance energy transfer (FRET) technique has been used to observe structural changes of α-tropomyosin, a coiled-coil protein, in aqueous solution[64]. In addition to his research, T. Yanagida serves on the editorial board of Single Molecules (the journal). The workings of a molecular motor, F_1-ATPase has been the subject of investigation by another group out of Japan. H. Noji, R. Yasuda, M. Yoshida, and K. Kinosita have directly observed the motion of single molecules of this motor and found it to rotate in discrete 120° steps, with an occasional reversal of directions, and have measured the work done in each step to be constant, independent of the load, and close to the free energy of hydrolysis of an ATP molecule, the fuel for this motor[65,66].

The FRET technique has also been used by Shimon Weiss and co-workers at the Lawrence Berkeley National Laboratory[67,68]. They attached two tetramethylrhodamine (TMR) molecules to a single staphylococcal nuclease molecule and observed energy transfer fluctuations between the two dye molecules caused by enzyme conformational dynamics on a millisecond time scale. They concluded that FRET is a powerful technique for studying conformational states and dynamics of biological macromolecules in various conditions: in equilibrium, during folding and unfolding, or during catalysis or ligand binding. In addition S. Weiss described identification methods, using FRET, for biomolecules freely diffusing through a detection region[69], and provided a nice overview of single molecule work to date and its future directions[70].

Many more groups have contributed to the field. Richard Zare, based on the work of Keller's group, speculated early on about the possibilities of the use of lasers in the single molecule studies[71] and later contributed to this field by studying the diffusive movements of single fluorescent molecules in solution[72]. He also performed studies on single enzyme molecules contained within biomimetic microcontainers where he attempted the manipulation of not the enzyme molecules, but rather of their nanoenvironment[73]. Rudolf Rigler and co-workers at the Karolinska Institute in Stockholm, Sweden, studied single molecules of horseradish peroxidase by fluorescence spectroscopy and found a wide distribution of enzymatic turnovers for the different molecules of this enzyme[74]. They also found activity fluctuations for the individual molecules on very short (sub-millisecond) time scales. To explain these, they evoked Frauenfelder's energy landscape description of protein folding[1,3], and postulated that the short time scale fluctuations are due to the enzyme experiencing different conformational substates.

As already stated, it would be impossible to provide a full review of the field of single molecule studies. For more information the reader is urged to turn to one of the special issues published on the topic, and previously mentioned.

1.2.4 Some Concepts in Single Molecule Enzymology

Single molecule enzymology differs from the traditional, classical enzymology in a few ways. Whereas the classical study of enzymes uses bulk measurements averaged over the many molecules of the enzyme that are present in the sample to characterize enzyme molecules, the new way uses the measurements performed on many individual molecules to describe the enzyme. Clearly, in the first approach all enzyme molecules look the same - average. The stochastic behaviour of individual molecules is lost within the ensemble average. It is only by performing single molecule studies that we can begin to appreciate that not all enzyme molecules are the same, and

that the "average" molecule of classical enzymology may not in fact even resemble the actual picture. A distribution of a certain property is certainly a better description than only its mean and standard deviation.

In the same vein, only single molecule studies can assist in the determination of static and dynamic disorder in enzymatic properties. Static disorder refers to the stationary heterogeneity of a property within a large ensemble of molecules. An example of static disorder is the heterogeneity in the activities of individual molecules of calf intestinal alkaline phosphatase as found by Craig et al.[41] or that found for lactate dehydrogenase molecules by Xue and Yeung[49](although the explanation provided by the authors, that of extremely slow fluctuations in enzyme conformation, would point to dynamic disorder on unusually long time scales). In addition, some of the findings of Lu *et al.*[58] and Edman *et al.*[74] also point to some degree of static disorder in their systems. The dynamic disorder on the other hand, is the time-dependent fluctuation of a property of a single molecule. An example of this type of disorder are the fluctuations over a range of time scales found by Lu *et al.*[58] in addition to the stationary heterogeneity in individual molecules activities. Dynamic disorder has also been observed by some of the other groups mentioned in the previous section[61,62,74]. Both static and dynamic disorder contribute to the broad distributions of molecular properties of ensemble systems. Two molecules can differ in their activities due to slight differences in their structures (glycosylation patterns, proteolytic patterns, amino acid substitutions at crucial sites), with these differences being stable on any time-scale. They can also differ due to various rates with which they fluctuate (by, for example, switching between various conformational substates in the protein energy landscape at different rates), with these differences normally restricted to short time scales. Determination of molecular property distribution would be exceedingly difficult by ensemble-average methods; differentiation between the static

and dynamic disorders would simply be impossible. Single molecule work makes both seem easy (relatively speaking of course).

Another advantage of performing studies on single molecules, when the measurements are done in real time, is the ability to detect the presence of transient intermediates whose concentrations may be too low for classical methods. Individual steps of the reaction can be often directly observed and can help us understand the reaction mechanism in greater detail. The findings of Ishijima *et al.*(62), discussed in the previous section, are an excellent example of how single molecule observations have changed the way some biological processes are viewed.

Yet another aspect specific to the study of single molecules is the issue of concentration. In classical enzyme kinetics we need to know the concentration of the enzyme being studied in order to determine such properties as specific activity or a turnover number. In single molecule studies, the concentration of enzyme becomes meaningless when discussing enzyme kinetics. We only ever deal with measurements done on a single molecule - the activity calculated for a single molecule is necessarily always specific. This "single molecule reasoning" becomes even more necessary when discussing the chemistry of a living cell. In these cells, the number of molecules of a particular enzyme may be very small under certain conditions(75). The concentration of an enzyme within a detection volume will then be a fluctuating quantity as the molecule diffuses in and out of this volume(60). An approach to studying reaction rates, known as fluctuation correlation spectroscopy, uses the analysis of such concentration fluctuations(76-79).

There are a number of difficulties associated with single molecule studies. One of them is the complexity of experimental set-up and its price. Most methods rely on fluorescence and the price of lasers or confocal microscopes can be prohibitive. Other methods use force microscopic techniques; this equipment is even more expensive. For the methods based on fluorescence, finding an appropriate system to work with can

also be a challenge. Most enzymes do not fluoresce strongly enough in their native form to be of use. Fluorescent or fluorogenic substrates must then be employed - naturally they cannot always be found. Contamination control can be challenging, especially with such an ubiquitous enzyme as alkaline phosphatase. Finally, an open mind-set is crucial. Single molecule experiments sometimes bring very surprising results and convincing the old establishment to accept the sometimes contradictory findings can be the greatest challenge of all.

1.3 ELECTROPHORESIS

Electrophoresis is the movement of electrically charged particles in a conductive liquid medium effected by the influence of an electric field. The rate of movement of various species is determined by their charges, sizes, and in certain situations their shapes, and the applied electric field. This rate is known as electrophoretic velocity and is described by the equation:

$$v_{EP} = \mu_{EP} E \qquad \textbf{Equation 1.3}$$

where μ_{EP} is the electrophoretic mobility and E is the applied electric field. Separation is possible because different solutes have different electrophoretic mobilities. Electrophoretic mobility can be described by the following equation:

$$\mu_{EP} = \frac{q}{6\pi\eta r} \qquad \textbf{Equation 1.4}$$

where q is the charge on the solute, η is the buffer viscosity, and r is the solute radius.

Electrophoresis is probably one of the most widely used techniques for the analytical separation of ions[80]. There are many modes of electrophoresis, depending on the support used. In this chapter I will limit the introduction to a discussion of gel electrophoretic techniques, extremely important in the study of biomolecules and utilized in the course of this research, and capillary zone electrophoresis, a newer technique which has found applications in many branches of life sciences. An

overview of a laser induced fluorescence - capillary electrophoresis (LIF-CE) instrument utilizing a sheath flow cuvette, as developed by N. J. Dovichi and used in the single enzyme molecule work described in **Section 1.2.2**, will also be provided.

1.3.1 Gel Electrophoresis

As the name suggests, gel electrophoresis is simply the electrophoresis of solutes in a gel matrix. It is a technique widely used with proteins and other biomolecules. In the simplest terms, a slab (or tube) gel is placed with its ends submerged in buffer(s), either vertically or in a horizontal position. The two buffers can be the same (a continuous buffer system) or they can be different (a discontinuous buffer system). A potential difference is applied to the ends of the gel which drives the charged molecules of the sample; the net charges on the molecules have an effect on their travel as described by **Equation 1.4**. These molecules then move through the pores of the gel which impede their movement based on the size of the molecules. The size of the pores is controlled by varying the gel composition.

Depending on the type of the gel medium, gel electrophoresis can be further divided into agarose gel electrophoresis, starch gel electrophoresis, and polyacrylamide gel electrophoresis (PAGE). The first technique is mainly employed for the separation of DNA fragments. The other two have been widely used in the study of proteins as well. Starch gel electrophoresis is no longer the preferred method in most cases due to the fragility of the gel and problems with densitometric staining[81].

PAGE can be also further subdivided, depending on the mode of separation. Native PAGE is a technique for separating proteins in their native state (non-denatured). It relies on the size and charge of a protein for separation. The gel serves as a molecular sieve whose pore size is controlled by the gel's composition (more accurately, by the ratio of acrylamide to bis-acrylamide, the two components that make

up polyacrylamide gels). There are a number of possible continuous and discontinuous buffer systems[81].

Sodium dodecylsulphate - PAGE (SDS-PAGE) is a technique used to separate proteins based solely on their molecular weights. This is achieved by denaturing the protein in the presence of a reducing agent, such as β-mercaptoethanol or DTT, and heat (96°C for 5 min. is a standard procedure), and then swamping its native charge with sodium dodecylsulphate, essentially converting every protein molecule into a highly negative rod-like particle. This leaves only the molecular size as the determining factor in electrophoresis. In addition, the denaturation procedure destroys any 4° and 3° structure characteristics - only monomeric subunits of proteins are detected. Naturally, enzymes treated in such ways are for most part catalytically dead although there have been reports of enzymatic activity recovery after SDS-PAGE[82]. Most proteins bind SDS in a uniform fashion - 1.4 g SDS per gram of reduced protein. There are some exceptions: papain, pepsin, glucose oxidase, and some glycoproteins bind relatively low amounts of SDS[83]. It is possible to use a continuous buffer system with SDS-PAGE but by far the most widely used is the discontinuous system of Laemmli[84].

Isoelectric focusing (IEF) is another method based on PAGE, although other types of gels have been used. In this technique the proteins are separated based on their net charge alone. The electrophoresis happens in a gel containing a mixture of polyampholytes which are small multi-charged molecules spanning a range of isoelectric points. An isoelectric point (pI) of a molecule is equal to the pH at which the net charge on the molecule is 0. The ampholytes can be either introduced with the running buffer or, in the newer commercial IEF gels, are immobilized right within the gel. The ampholytes are used to set up a pH gradient through which the protein travels under the influence of an electric field. Once the protein reaches a place in the gel where the pH is equal to its pI, the net charge on the protein becomes zero and the

movement of the molecule stops. Any diffusion is prevented by the movement of the molecule back into the neutral zone and so bands obtained by IEF can be extremely narrow. Resolution of 0.01 pI units is not uncommon. The moment the electric field is removed however, the diffusion of sample molecules begins and so immediate processing of the gel is crucial. IEF can be performed in PA gels containing urea, which can denature the proteins, or it can be done without it, leaving the proteins intact.

The final example of PAGE is two dimensional gel electrophoresis (2-D PAGE). This is easily today's most powerful tool for separation of complex protein mixtures, such as cell extracts. This technique is the marriage of IEF and SDS-PAGE, or native PAGE and IEF, resulting in denaturing or non-denaturing conditions, respectively. In most commonly performed 2-D PAGE, a sample is first applied to the IEF gel, isoelectrically focused, and then the IEF gel is manually placed on top of an SDS-PA gel and the proteins are allowed to elute out of the first dimension matrix and into the second dimension SDS-PA gel where separation according to MW takes place. An equilibration step is required in between the two dimensions to allow for the denaturation and coating of the protein with SDS. After the second dimension the contents of the gel are visualized, appearing as a collection of spots and smears. These can then be removed and studied, most often by matrix assisted laser desorption ionization mass spectrometry (MALDI-MS). The combination of 2-D PAGE and MALDI-MS is the driving force behind proteomics research today.

A final word in this section must be said about protein visualization. Many methods exist for staining gels. The most common ones are silver staining and Coommassie blue staining. The first of the two is more sensitive but also more expensive, wasteful, and environmentally damaging due to the use of silver ions. It also does not work for all the proteins equally well. Coomassie staining takes a little longer and its about 10 times less sensitive but is also more universal and easier. Other staining methods of interest here are Zn staining[85-87], a reverse staining technique in

which the gel, not the protein within it, is stained, and activity based staining in which substrates for enzymes can be employed to bring out the sample.

1.3.2 Capillary Electrophoresis

Capillary electrophoresis is the movement of charged species inside a narrow bore capillary under the influence of an electric field. The technique was first described by Jorgenson and Lukacs in 1981[88-90]. The basic principle of electrophoretic mobility of solutes as described in **Equation 1.4** still applies but the observed electrophoretic mobility of a solute is determined by another factor, the electroosmotic mobility. Electroosmosis is a phenomenon unique to capillary electrophoresis. The electroosmotic flow (EOF) occurs as a result of the capillary's wall having an overall negative charge when a buffer is placed within it, causing the formation of the so called diffuse double layer of positively charged cations. The outer part of the layer is pulled towards the negative end of the capillary when the electric charge is applied causing a net movement of the buffer in that direction. The electrophoretic mobility is almost solely dependent on the buffer characteristics according to the equation:

$$\mu_{EOF} = \frac{\varepsilon\zeta}{4\pi\eta} = \frac{\delta e}{\eta} \qquad \textbf{Equation 1.5}$$

where ε is the dielectric constant of the buffer, ζ is the zeta potential, η is the viscosity of the buffer, δ is the thickness of the double layer, and e is the charge per unit surface area of the capillary wall. The thickness of the double layer, δ is inversely related to buffer concentration. The magnitude of the electroosmotic mobility of the buffer is normally greater than the electrophoretic mobilities of the solutes causing all species in the capillary to move in the same direction. The observed electrophoretic mobility of a species in the presence of EOF is the sum of the electrophoretic mobilities of the solute and the buffer. Thanks to EOF capillary electrophoresis is able to simultaneously resolve positive, negative, and neutral species in a single run. EOF can be manipulated

by adjusting any of the buffer characteristics described in the equation above, or by modifying the capillary surface through, for example, coating.

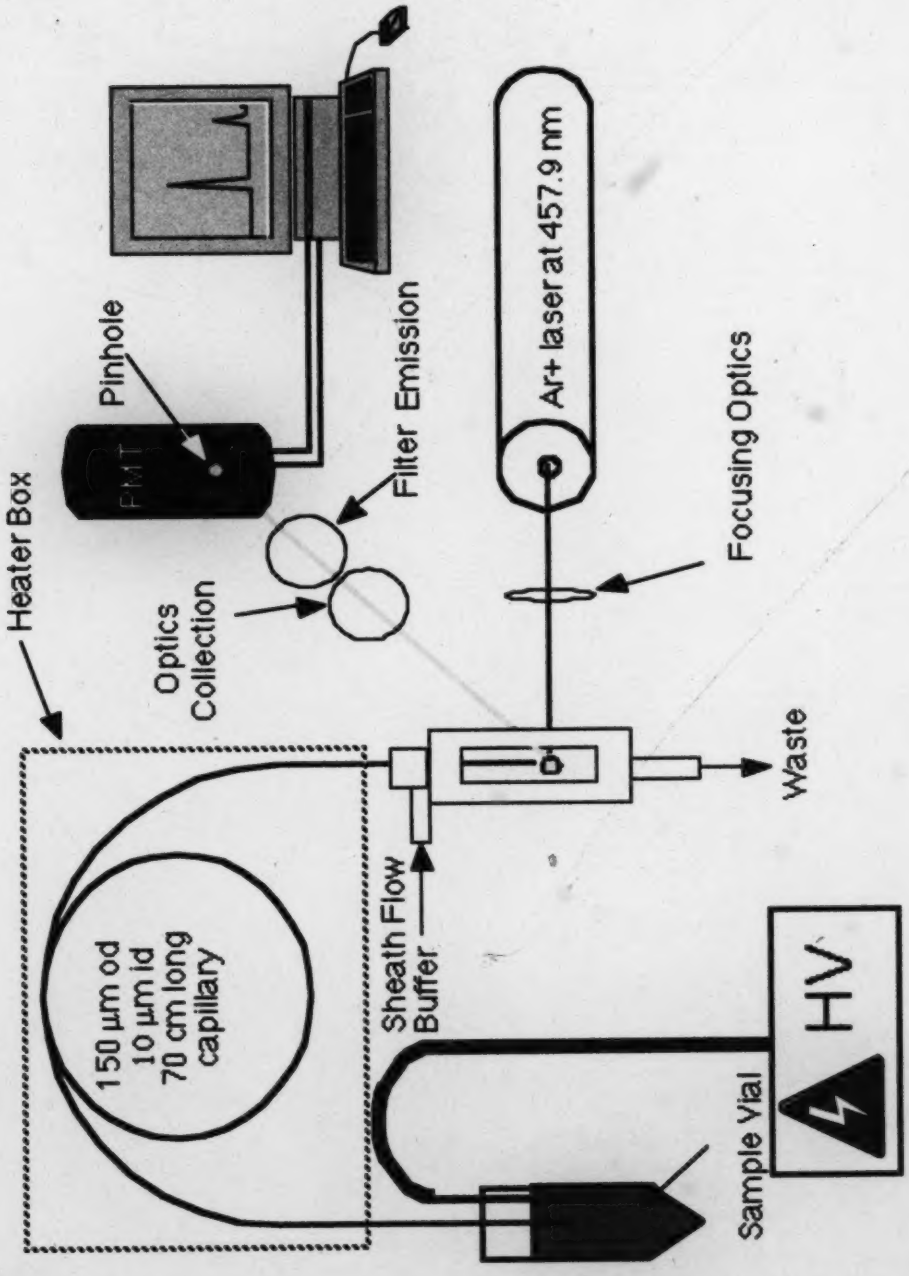
In CE experiments conducted on single molecules in our research group EOF is responsible for the separation of the enzyme, the substrate and the product of the reaction. At the pH of the borate buffer (9.5) the electrophoretic mobility is the highest for the substrate, a small molecule carrying a double negative charge, followed by the product, yet a smaller molecule with a single negative charge, and finally the enzyme, a very large molecule whose multiple negative charges are negated by the size. With the positive injection end, in the absence of the EOF, none of these molecules would be expected to enter the capillary. EOF however, draws them in, reversing their observed electrophoretic mobilities so that the enzyme moves first, followed by the product, and finally the substrate. Modification of the EOF is achieved by the inclusion of an organic modifier (DMSO) with the borate buffer.

1.3.3 CE-LIF Instrument Utilizing a Sheath-Flow Cuvette

The details of the CE-LIF instrument utilizing a sheath-flow cuvette, as used extensively in our laboratory, have been published previously[29]. The instrument as used by Craig *et al.* in his work with single molecules of alkaline phosphatase will be described here. Changes made to the experimental set-up will be explained in **Section 2.3.2.1**. A schematic diagram of the instrument is shown in **Figure 1.2**.

A 10 μm i.d., 150 μ.m. o.d. fused silica capillary of variable length is used as a reaction vessel. The capillary has its polyimide coating removed at the detection end. The detection end is contained inside a in-house designed sheath-flow cuvette while the injection end is placed inside a plastic 0.6 mL tube containing the sample. A platinum wire electrode is submerged in the same tube and is connected via high voltage cable to a Spellman 1000 power supply (Spellman, Plainview, NY) set to positive polarity injection. The sheath-flow cuvette is connected to a sheath-flow buffer reservoir and

Figure 1.2 A schematic diagram of a CE-LIF set-up as used in the work of Craig *et al.* and later in the course of my research. The heater unit was not originally present but added later as described in **Section 2.3.2.1.**



has two lines out: one for waste and the other for manual purging. The sheath-flow buffer reservoir is placed at 3 cm above the waste container for optimal sheath flow. The sheath-flow cuvette is grounded. Monochromatic light for excitation is provided by a tunable CW Ar^+ laser (Innova 99, Innova Corp., Palo Alto, CA) set to 457.9 nm and 20 mW constant power output. The light from the laser is deflected by two mirrors at 90° and it passes through a 4 × neutral density (N.D.) filter and a 1 mm pinhole before being focused with a 6.3 × microscope objective (Melles Griot, Nepean, ON) into the cuvette about 20 μm below the exit of the capillary. Fluorescence from the eluting species is collected at 90° with a 60 × microscope objective (Melles Griot) with a numerical aperture of 0.70 and passed through another pinhole and a 580 dF 40 band pass filter before being focused onto a photomultiplier tube (PMT) (Hamamatsu R1477, Osaka, Japan). Electrical signal from the PMT is collected in LabView on a MacIntosh IIsi computer.

1.4 THESIS SUMMARY

This thesis describes work done in the area of single molecule enzymology with alkaline phosphatase as a model enzyme. Capillary electrophoresis with laser induced fluorescence, and utilizing a sheath-flow cuvette, is the method of choice for the assessment of enzyme characteristics such as activity, activity-structure relationship, kinetic behaviour, and thermal stability. In addition, methods used in enzyme sample preparation, bulk enzyme sample assessment (classical enzymology), and instrumental design changes are described. The three experimental chapters of this thesis are not arranged in chronological order.

Chapter 2 deals with the structure - enzymatic activity relationship and the thermal denaturation of an enzyme based on the single molecule studies of *E. coli* alkaline phosphatase. The introduction provides a literature overview of our knowledge of this particular alkaline phosphatase isozyme. The next section deals with

initial assessment of a commercial preparation of this enzyme. Evidence is given for great structural and functional heterogeneity of *E. coli* alkaline phosphatase. Methods used in the preparation of active isoforms of this enzyme, and improvements to the instrument, designed to shorten assay time, are described next. The following section deals with the on-capillary single enzyme molecule assays and provides answers for the observed heterogeneity of single molecule activities. Thermal denaturation study of *E. coli* alkaline phosphatase, presented in **Section 2.5**, tries to answer the question of the denaturation pathway taken by the enzyme. Conclusions are offered at the end of the chapter.

Chapter 3 deals with a few separate issues. The introduction provides some general information on alkaline phosphatase as well as details of alkaline phosphatases from calf intestine, bovine kidney, porcine kidney, human placenta, and shrimp. In addition some considerations of the instrumental and assay design, and of enzyme type are outlined. In the next section some of the first attempts at single enzyme molecule work, undertaken as a continuation of the work done previously in this laboratory by Craig *et al.*[41-43], are described. Attempts at better understanding the relationship between calf intestinal alkaline phosphatase glycosylation patterns and the great heterogeneity previously observed in the activities of single molecules of this enzyme are outlined. These include the efforts in purifying the isoforms, in deglycosylating the enzyme, and in improving assay conditions to better suit the enzyme and to improve sensitivity. The failure to fulfill these goals led to a change in model enzyme and, in turn, to the work described in the previous chapter. In the following section, single molecule and classical bulk enzyme solution work on the remaining four alkaline phosphatases is described. Conclusions are offered at the end of the chapter.

Chapter 4 deals with the kinetics of single enzyme molecules. It describes the work done on immobilized molecules of calf intestinal alkaline phosphatase. In the introduction, the concept of enzyme immobilization is outlined. Following that is the

description of immobilization procedure and the preparation of a fused silica capillary - immobilized enzyme reactor for use with CE. Section 3 describes the methods used in the study of immobilized alkaline phosphatase, the results obtained, and provides a discussion of the immobilization effects on the kinetic constants by comparison with assays of free enzyme in bulk solution. Some conclusions wrap up the chapter.

Chapter 5 sums up the work done in the course of my research and provides some thoughts for the future of single molecule studies on alkaline phosphatase.

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2

Studies on Single Molecules of *E. coli* Alkaline Phosphatase

2.1 INTRODUCTION

The alkaline phosphatase of *E. coli* is one of the better known, and possibly the best characterized isoenzyme of the alkaline phosphatase family. It has been purified[1-3], crystallized[2,4,5], and sequenced[6] and thus its structure and amino acid sequence are known[7]. *E. coli* alkaline phosphatase is the product of a single gene, *pho*A[8]. It is a dimer of 450/449 amino acids[5], with the molecular weight of 89 kDa[9,10]. Each monomer contains 2 Zn^{2+}[10] and 1 Mg^{2+}[10], both essential to its activity, as well as one phosphate molecule[11]. Its active site contains the amino acid triplet Asp-Ser-Ala, analogous to all other alkaline phosphatases[12]; the active Ser residue appears to be also shared by most hydrolases. The structure and the amino acid sequence of *E. coli* alkaline phosphatase subunit are shown in **Figures 2.1** and **2.2.**

The enzyme of an *E. coli* bacterium is present in the periplasmic space[5,13], between the cell wall and the plasma membrane. The enzyme, which is synthesized at the ribosomes as an apoenzyme, must be transported to its final destination. A 21 amino acid, highly hydrophobic signal sequence at the amino-terminus assists in this task. Once in the periplasmic space, the signal sequence is cleaved off via the proteolytic action of the product of the *iap* gene[14]. The enzyme is then folded, it aquires its cofactors, and it is dimerized[15]. In contrast to most other alkaline phosphatases, the *E. coli* isoform is not extensively post-translationally modified - it contains no sugars and no lipids on its surface[5]. Under normal growth conditions, *E. coli* may posses as little as a single alkaline phosphatase molecule per cell[16]. When grown under conditions of phosphate starvation however, the production of alkaline phosphatase is highly stimulated, resulting in as much as 6% of total protein content of the cell[1].

The dimerization of alkaline phosphatase in *E. coli* accounts for the observed three bacterial isoforms. The difference between the subunits is due to the cleavage of the N-terminal Arg of a processed enzyme. As already mentioned, the apoenzyme is

Figure 2.1 A ribbon diagram of an *E. coli* alkaline phosphatase subunit showing the metal cofactors (2 zinc and 1 magnesium ions), a phosphate molecule, three water molecules and two disulfide bridges. Adapted from Expasy's Swiss-3D Image database at http://expasy.cbr.nrc.ca/sw3d/, accession number P00634, file S3D00045.rgb.

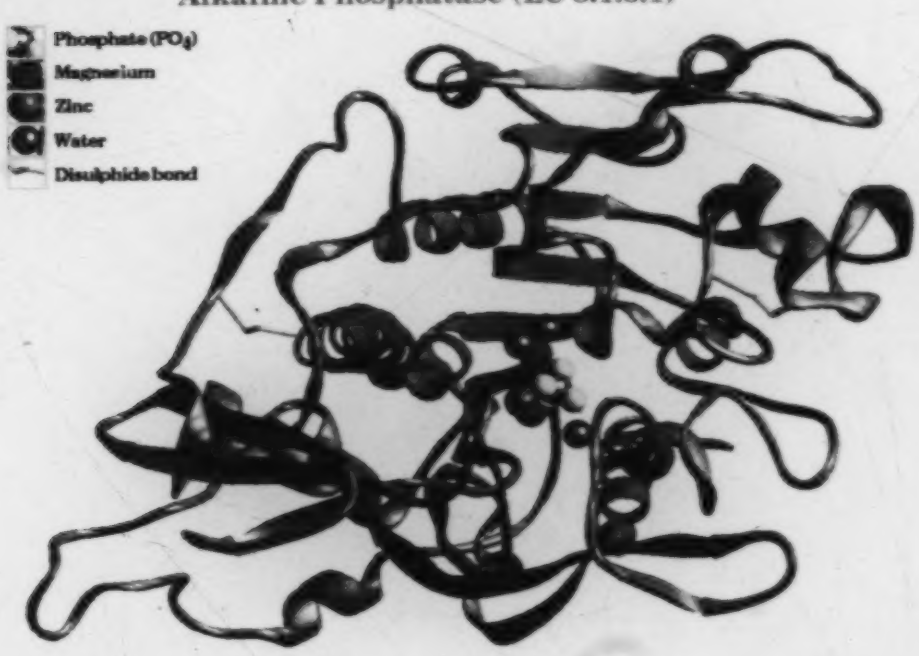


Figure 2.2 The amino acid sequence of an E. coli alkaline phosphatase subunit[6], including the signal sequence, starting at the N-terminus. Adapted from Expasy's Swiss-Prot database at http://expasy.cbr.nrc.ca/

M(1) - A(21) = signal sequence

R(22) = N-terminal Arg of apoenzyme, missing in isoform 3

S(124) = active site, phosphorylation site

G(72), **D**(175), **T**(177), **E**(344) = coordinated to Mg

D(349), **H**(353), **H**(392), **H**(394) = coordinated to Zn1

D(73), **D**(391), **H**(392) = coordinated to Zn2

C(190)/**C**(200), and **C**(308)/**C**(358) - disulfide bridges

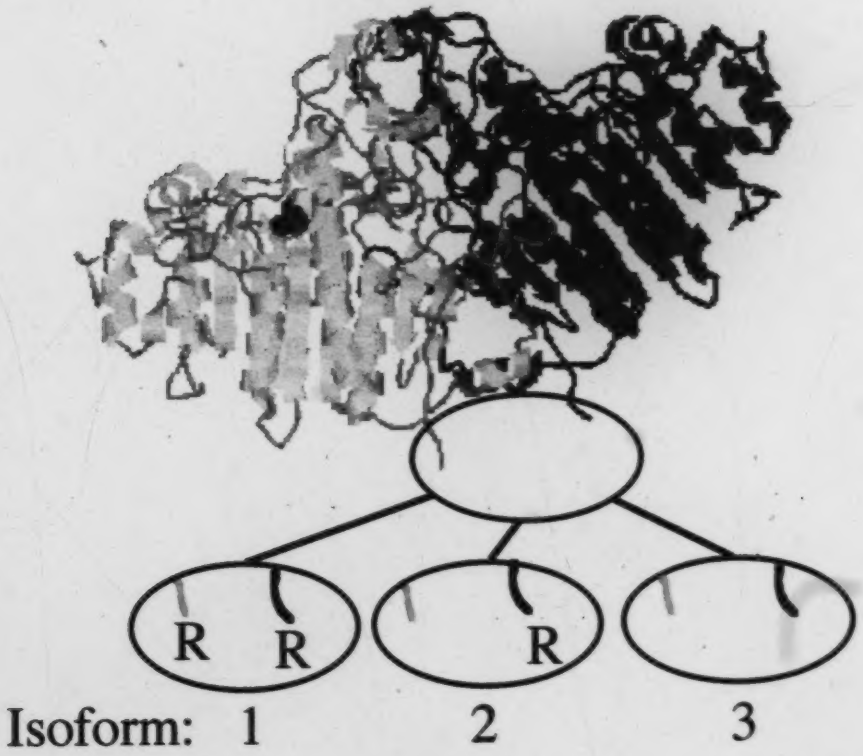
```
          10         20         30         40         50
           |          |          |          |          |
MKQSTIALAL LPLLFTPVTK ARTPEMPVLE NRAAQGDITA PGGARRLTGD
          60         70         80         90         100
           |          |          |          |          |
QTAALRDSLS DKPAKNIILL IGDGMGDSEI TAARNYAEGA GGFFKGIDAL
         110        120        130        140        150
           |          |          |          |          |
PLTGQYTHYA LNKKTGKPDY VTDSAASATA WSTGVKTYNG ALGVDIHEKD
         160        170        180        190        200
           |          |          |          |          |
HPTILEMAKA AGLATGNVST AELQDATPAA LVAHVTSRKC YGPSATSEKC
         210        220        230        240        250
           |          |          |          |          |
PGNALEKGGK GSITEQLLNA RADVTLGGGA KTFAETATAG EWQGKTLREQ
         260        270        280        290        300
           |          |          |          |          |
AQARGYQLVS DAASLNSVTE ANQQKPLLGL FADGNMPVRW LGPKATYHGN
         310        320        330        340        350
           |          |          |          |          |
IDKPAVTCTP NPQRNDSVPT LAQMTDKAIE LLSKNEKGFF LQVEGASIDK
         360        370        380        390        400
           |          |          |          |          |
QDHAANPCGQ IGETVDLDEA VQRALEFAKK EGNTLVIVTA DHAHASQIVA
         410        420        430        440        450
           |          |          |          |          |
PDTKAPGLTQ ALNTKDGAVM VMSYGNSEED SQEHTGSQLR IAAYGPHAAN
         460        470
           |          |
VVGLTDQTDL FYTMKAALGL K
```

acted upon by a protease, a product of the *iap* gene, after being transported into the periplasmic space. It appears, that the same protease can remove the now-terminal Arg[14]. As a result, monomers are produced of which some will contain the full 450 amino acid sequence while others are missing one Arg. Upon dimerization three different "native" isoforms are obtained - two homodimers, one with both terminal Arg, and one without either, and one heterodimer possessing just one terminal Arg[17](see **Fig. 2.3**). Since Arg carries a positive charge, its loss affects the isoelectric point (pI) of the enzyme allowing for the easy separation of the isoforms by isoelectric focusing[18]. In the presence of ampholytes in an electric field, the isoforms will fractionate starting with the Arg homodimer at the cathodic end and ending with the Arg-free homodimer at the anodic end. The heterodimer migrates in the middle. For the purpose of this work, the accepted designation of isoforms as 1,2, and 3, starting from the cathodic end, will be employed[16]. The approximate pI values for the three isoforms fall between 5.5 and 5.1[18].

Two of the more distinguishing features of the *E. coli* isozyme of alkaline phosphatase are its comparatively low pH optimum and comparatively high temperature stability. The pH optimum is around 8.0 while the enzymatic activity appears to increase with temperature up to 80°C[1]. By comparison, all mammalian and most other alkaline phosphatases exhibit pH optima around 10.0-11.0 and temperature optima below 50°C [19].

In comparison to the calf intestinal alkaline phosphatase, which had been extensively used in this laboratory by Craig *et al.* to develop the single molecule on-capillary assays[20-22], the *E. coli* isozyme is a much simpler and robust enzyme, although not nearly as active. Since it is relatively easy to fractionate by IEF, it is possible to isolate it in large enough quantity to perform further assays. These single molecule assays should then help in better understanding the relationship between the structure and function of enzyme molecules.

Figure 2.3 A ribbon diagram of E. coli alkaline phosphatase dimer. The two subunits are designated by different shading. The differences between isozymes are exaggerated. R = arginine. Adapted from Expasy's Swiss-3D Image database at http://expasy.cbr.nrc.ca/sw3d/, accession number P00634.



In addition to exploring functional heterogeneity of calf intestinal alkaline phosphatase, Craig *et al.*[20-22] also looked at potential thermal denaturation pathways for that enzyme. Two possible models were proposed for the thermal denaturation and the death of enzyme molecules. In the first model, the "Cheshire Cat" model, individual molecules of enzyme go through a number of changing conformations at the secondary level of structure; these conformations have progressively lower activities ending with fully denatured and inactive molecules. Thus the overall gradual decrease in activity of a bulk enzyme sample is a result of comparable decreases in activities of individual molecules. In contrast, the catastrophic model proposes abrupt losses of activity when the active site of a molecule undergoing denaturation is damaged. Small conformational changes along this pathway have no effect on the activity of the molecules. In this model the overall gradual decrease in activity of a bulk sample is due to progressively larger fraction of molecules losing all of their activity while the remaining fraction survives intact. For the calf intestinal alkaline phosphatase, Craig *et al.*[20] showed that the enzyme denatures according to the second, catastrophic, model. An analogous study of thermal denaturation is reported here for *E. coli* alkaline phosphatase. This enzyme has previously been shown to be very heat stable[1,15]. In crude extracts heated at 85°C for 30 min., no activity was lost. Purified enzyme was not as stable, but in the presence of Mg^{2+} it retained 50% of its activity even after 8 min. at 95°C. Furthermore, heat-inactivated enzyme was able to recover most of its activity when cooled prior to assays. In these respects, the *E. coli* isoform is unique among alkaline phosphatases.

2.2 INITIAL STUDIES OF *E. COLI* ALKALINE PHOSPHATASE

Although the *E. coli* isoform of alkaline phosphatase had been previously used in the single molecule assays in this group, a very limited amount of work was done on it. This was mainly due to the low activity of the enzyme, especially under the assay

conditions employed. To assess the suitability of the enzyme for future single molecule work, the enzyme samples were assayed by single molecule on-capillary assays and were run on SDS-PAGE, 2D-electrophoresis gels, and IEF gels. Initial studies by the author were undertaken on two commercial preparations of *E. coli* alkaline phosphatase. Preparation 1 (Sigma-Aldrich Canada Ltd., Oakville, ON, cat.# P 4252) was Type III *E. coli* alkaline phosphatase, chromatographically purified, a suspension in 2.5 M $(NH_4)_2SO_4$ solution, with an activity of 30-90 units per mg of protein, where one unit is the amount of enzyme that will hydrolyze 1.0 µmol of p-nitrophenyl phosphate per min. at 37°C. Preparation 2 (also from Sigma, cat.# P 4069) was *E. coli* alkaline phosphatase solution in 50% glycerol containing 5 mM Tris HCl, 0.5 mM $MgCl_2$, and 0.5 mM $ZnCl_2$, pH 7.4, with an activity of 20-50 units per mg of protein, where the unit definition is as for Preparation 1.

2.2.1 Isoelectric Focusing of *E. coli* Alkaline Phosphatase

Prior to IEF, Preparations 1 and 2 were processed to improve electrophoresis. Preparation 1 contained 2.5 M $(NH_4)_2SO_4$, an amount of salt that could potentially interfere with the electric field during focusing. IEF performed initially with this sample on a horizontal bed IEF instrument, a Pharmacia PhastSystem (Pharmacia LKB, Uppsala, Sweden), resulted in damage to the instrument due to salt precipitate causing an electrical short between the gel and the electrode. Therefore subsequent samples of this preparation were desalted either by batch dialysis using Pierce Slide-A-Lyzer® Dialysis Cassettes, 10,000 MWCO, 0.1-0.5 mL capacity (Chromatographic Specialties, Brockville, ON) or by micro-spot dialysis on floating Amicon 10,000 MWCO membranes (Millipore, Bedford, MA). In either case the dialysis was against 50% glycerol. At that point, Preparation 1 and Preparation 2 were diluted in ddH_2O prior to application onto the Pharmacia PhastSystem 8/1 sample comb at 1-2 µL/lane. The dilution is necessary because the high viscosity of glycerol impedes the movement

of the sample on the horizontal bed (this is not an issue when using vertical IEF however). The application comb was loaded in the central or anodic position to allow sufficient distance for the focusing. In cases when the comb was loaded in the central position, i.e. closer to pI of the enzyme, protein precipitation at the loading point was sometimes observed (see **Figure 2.4b**). Both samples were then run on Pharmacia PhastGels IEF 4-6.5 media (Amersham Pharmacia Biotech, Uppsala, Sweden), using a Pharmacia PhastSystem and the Separation Technique File No. 100 optimized for the pH gradient used:

Step 1: 2000 V, 2.0 mA, 3.5 W, 15°C, 75 Vhr - prefocusing

Step 2: 200 V, 2.0 mA, 3.5 W, 15°C, 15 Vhr – sample application

Step 3: 2000 V, 5.0 mA, 3.5 W, 15°C, 410 Vhr – sample focusing.

The standards used for IEF were from the Pharmacia IEF Low pI Calibration Kit (Pharmacia Biotech, Uppsala, Sweden) which consists of the following proteins (isoelectric points given in brackets): pepsinogen (2.80), amyloglucosidase (3.50), glucose oxidase (4.15), soybean trypsin inhibitor (4.55), ß-lactoglobulin A (5.20), bovine carbonic anhydrase B (5.85), human carbonic anhydrase B (6.55). The first 2 standards do not show up as distinct bands on the pH 4-6.5 gradient gels, but rather as a single band at the anodic end of the gel.

The gels were then silver-stained using the following modification of Pharmacia PhastSystem Development Technique File No. 210 (each step represents a change of solution):

Step 1 – fix: 20% TCA, 5 min., 20°C

Step 2 - wash: 50% EtOH / 10% HAc, 2 min., 50°C

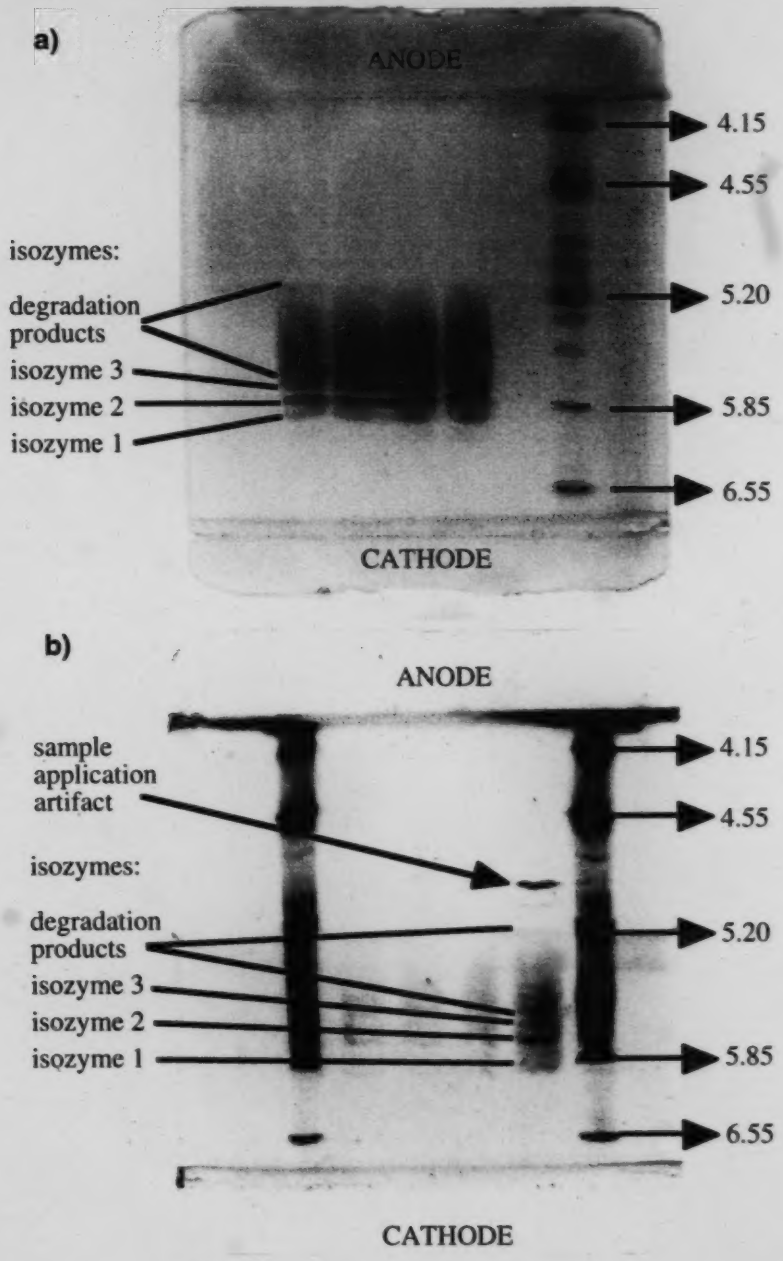
Step 3 - wash: 10% EtOH / 5% HAc, 2 min., 50°C

Step 4 - wash: 10% EtOH / 5% HAc, 4 min., 50°C

Step 5 - sensitizer: 8.3% glutardialdehyde, 6 min., 50°C

Step 6 – wash: 10% EtOH / 5% HAc, 3 min., 50°C

Figure 2.4 Isoelectric focusing of *E. coli* alkaline phosphatase: **(a)** Preparation 1, **(b)** Preparation 2 (see **Section 2.2**). The samples were run on a Pharmacia PhastSystem using Pharmacia PhastGel IEF, pH 4-6.5, and stained using a modification of Pharmacia silver staining protocol (see **Section 2.2.1**).



Step 7 – wash: 10% EtOH / 5% HAc, 5 min., 50°C

Step 8 – wash: ddH_2O, 2 min., 50°C

Step 9 – wash: ddH_2O, 2 min., 50°C

Step 10 – stain: 0.5% $AgNO_3$, 10 min., 40°C

Step 11 – wash: ddH_2O, 30 sec., 30°C

Step 12 – wash: ddH_2O, 30 sec., 30°C

Step 13 – developer: 0.04% formaldehyde / 2.5% Na_2CO_3, 30 sec., 30°C

Step 14 – developer: 0.04% formaldehyde / 2.5% Na_2CO_3, 4.5 min., 30°C

Step 15 – stop: 5% HAc, 7 min., 50°C

Once stained, the gels air-dry very quickly and are easily stored.

2.2.1.1 Evidence for Alkaline Phosphatase Heterogeneity by IEF

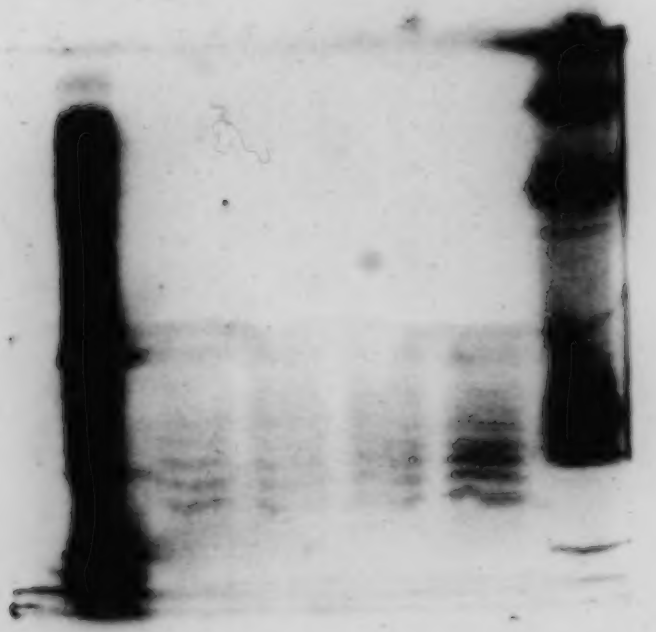
Both preparation were found to be quite heterogeneous by IEF where at least 9 bands were observed when using Preparation 1 and at least 7 bands when using Preparation 2 (see **Fig. 2.4**). Clearly, with only three "native" isoforms expected from purified *E. coli* alkaline phosphatase, the remaining bands must be due to some additional processing of the enzyme. In the case of *E. coli* alkaline phosphatase, devoid of sugars or lipids, this processing would most likely be a further proteolytic cleavage by either the product of the *iap* gene (see **Section 2.1**), or another protease present in the periplasmic space. This cleavage, if proceeding in a step-wise fashion, with only one to a few amino acids removed at a time, may produce a number of enzymatically active species before the activity is lost[23,24]. In addition, after the loss of activity, the proteolytic cleavage would certainly produce even more species. In a commercial preparation, many of these active and inactive enzymatic species could be expected to pass through the purification procedure along with the actual enzyme. All these could be expected to be seen in IEF gels developed with silver- or Coomassie-based stains. It is of course possible that this processing occurs not in the cell but

rather is a result of contamination with proteases during purification, or that contamination occurred upon arrival and digestion upon storage of the commercial preparation (a slow process at 4°C but certainly damaging when coupled with frequent warming/cooling of the enzyme solution). Although I have previously noted proteolytic degradation of stored commercial preparations of both alkaline phosphatase and other enzymes, when a freshly obtained Preparation 1 was separated by IEF, there were already more than three species present (see **Fig. 2.5**). This suggests that the enzyme may already be somewhat degraded prior to shipment, and hence likely not fully free of proteases.

2.2.2 SDS-PAGE AND 2D-GEL ELECTROPHORESIS OF *E. COLI* ALKALINE PHOSPHATASE

Both commercial preparations were also separated by SDS-PAGE and by 2D-gel electrophoresis to assess molecular weight homogeneity and to observe any other uncovered artifacts. For SDS-PAGE the samples were run on 12% separating / 4% stacking, 1 mm thick gels using a BioRad MiniProtean II vertical gel electrophoresis cell (Bio-Rad Laboratories, Hercules, CA), at 200 V constant voltage, for a total run time of about 35 min., monitored by the movement of tracking dye. Tris/Glycine/SDS buffer system was employed. For 2D-gel electrophoresis the samples were first run in IEF tube gels using a procedure modified from BioRad 2D-gel electrophoresis protocol (see recipe in **Section 2.2.2.1**). For the second dimension, the tubes were run on SDS-PAGE gels of the same composition as above but having a single well for standards and a long well for the 1st dimension tube gel. A number of different standards were employed in these runs. For the IEF in tube gels, BioRad Prestained IEF Standards were used in a separate tube run alongside samples to assess the progress of focusing. For the 2nd dimension of and for SDS-PAGE of Preparation 1, BioRad Kaleidoscope Standards were employed. For the SDS-PAGE of Preparation 2

Figure 2.5 Isoelectric focusing of a commercial preparation of E. coli alkaline phosphatase. A new stock of Preparation 1 (see **Section 2.2**) was received on 16.12.1998 and ran on the same day on a Pharmacia PhastGel IEF pH 4-6.5 media using the PhastSystem and stained using a modification of Pharmacia silver staining protocol (see **Section 2.2.1**). The standards were overloaded resulting in dark smears upon staining. The sample is in the middle four lanes.



BioRad Silver Stain Low Range SDS-PAGE Standards were used. On other occasions, NEB Prestained Protein Markers (New England BioLabs Inc., Beverly, MA) or BioRad Prestained SDS-PAGE Standards, were also used. After electrophoresis SDS-PA gels were stained using BioRad's Silver Stain Plus.

2.2.2.1 Instructions for IEF Gel Casting and 1st Dimension IEF

Tube gel composition (10 mL of solution):

- 5.50 g urea
- 2.00 mL 10% Triton® X-100 (deionized with AF 501-X8 ion exchange resin)
- 1.81 mL ddH2O
- 0.950 mL 40% acrylamide
- 0.540 mL 4% bis-acrylamide
- 0.400 mL BioRad 5/7 ampholyte solution
- 0.100 mL BioRad 3/10 ampholyte solution
- 33 µL 10% APS (freshly made)
- 7 µL TEMED

Mix all but the last two components at T<45°C. Add APS and then, immediately, TEMED; stir gently. Arrange tube gels in a glass vial. Pour gel solution into the vial until the tubes are covered. Cover the vial and allow gel to polymerize for at least 30 min. When the gel is set, push enough out one end, using a syringe, to allow for sample (about 1.5 cm). Prepare sample by mixing with equal volume of sample buffer (9.5 M urea, 2% Triton® X-100, 5% β-mercaptoethanol, 1.6% BioRad 5/7 ampholyte solution, 0.4% BioRad 3/10 ampholyte solution). After loading onto gels, add sample overlay buffer (3 M urea, 0.27 % BioRad 5/7 ampholyte solution, 0.07% BioRad 3/10 ampholyte solution, 0.0008% bromophenol blue). Use 100 mM NaOH as the cathode buffer (upper chamber) and 10 mM H_3PO_4 as the anode buffer (lower chamber). Run a 10 min. pre-focusing step at 500 V and allow about 2-2.5 hrs. at 750 V for IEF.

Monitor the progress of IEF by running a tube of BioRad Prestained IEF Standards parallel to sample tubes, and by observing the tracking dye in the sample overlay buffer.

2.2.2.2 Further Evidence for Alkaline Phosphatase Heterogeneity

SDS-PAGE and 2D-electrophoresis provided further evidence for the heterogeneity of commercial preparations of E. coli alkaline phosphatase. SDS-PAGE resulted in a number of bands in each case.

Preparation 2 showed at least 8 bands: the expected band at approximately 45 kDa, and a number of bands at lower MW, of which the most prominent is a band at approx. 20 kDa (see **Fig. 2.6**). The lower MW bands point to the degradation of the main subunit; this degradation is too great to be explained by the differences in the structures of isoforms, i.e. the removal of the N-terminal Arg. One possible explanation for these bands could be degradation during heat denaturation prior to SDS-PAGE; however, when a sample was not heated in order to observe this possible effect, a number of bands at lower MW still showed up. This points to degradation of enzyme upon storage or prior to arrival as the source of the high number of bands.

Preparation 1 in particular showed a minimum of 14 bands below the expected band (see **Fig. 2.7**). Similarly to IEF results, where this preparation seemed to result in greater number of bands than Preparation 2, Preparation 1 appears again to be more degraded. Absence of significant bands above the monomer's molecular weight suggests that all bands come from the degradation of subunits.

2D-gel electrophoresis results were obtained for Preparation 2 but not for Preparation 1 where the sample failed to focus in the first dimension, possibly because of high salt content of the sample. 2D-gel showed a number of spots including at least 3 at the expected MW, at least 2 at approx. 29 kDa, and at least 7 more at various MWs

Figure 2.6 SDS-PAGE of *E. coli* alkaline phosphatase Preparation 2 (see **Section 2.2.1**). The standards used were Bio-Rad Silver Stain SDS-PAGE Low Range Standards, and included: rabbit muscle phosphorylase b (97.4 kDa), BSA (66.2 kDa), hen egg white ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), hen egg white lysozyme (14.4 kDa).

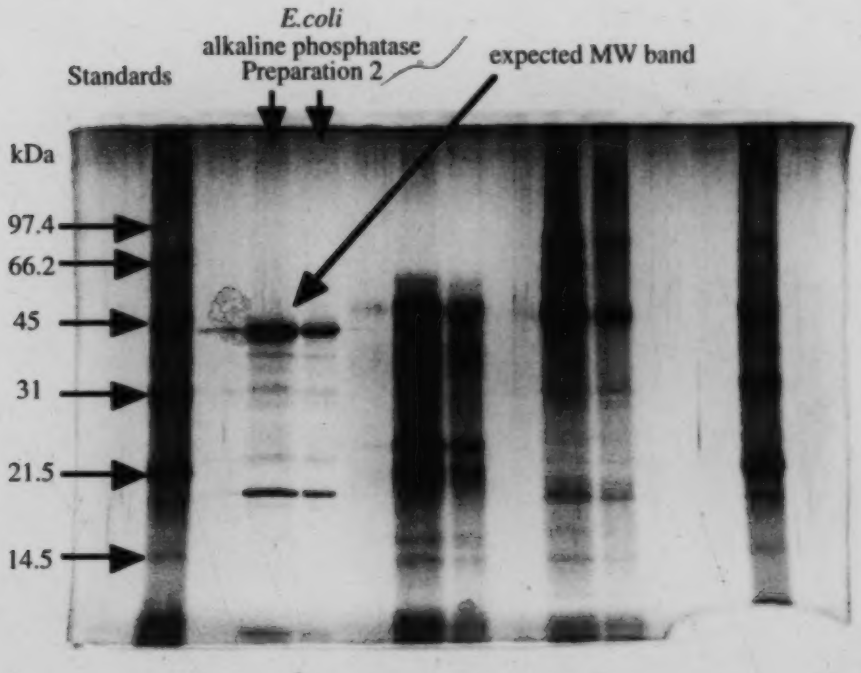
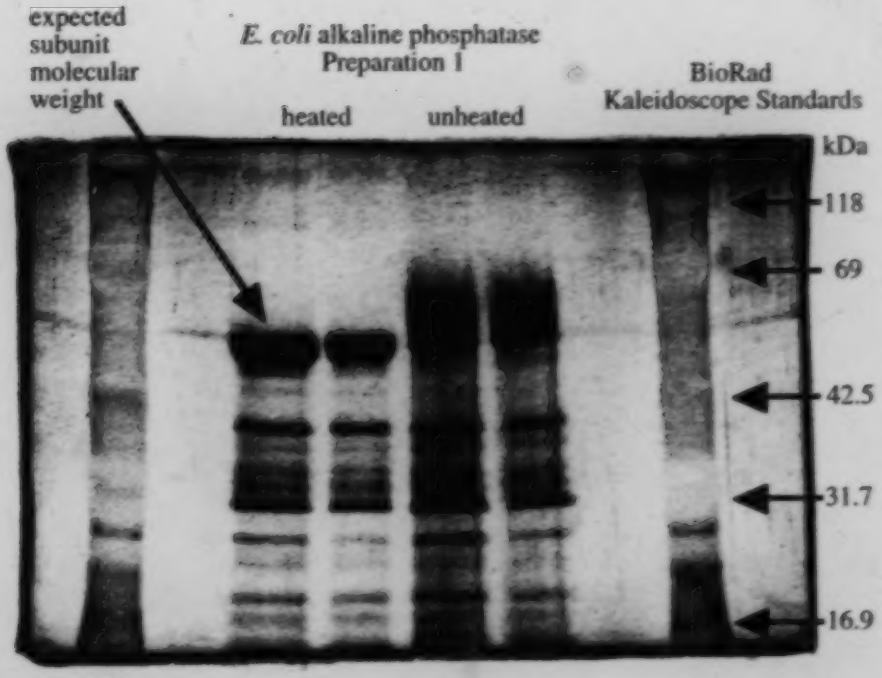


Figure 2.7 SDS-PAGE of E. coli alkaline phosphatase Preparation 1 (see **Section 2.2.1**). BioRad Kaleidoscope MW markers were used; because they do not stain with silver, their positions are indicated with arrows. The markers are: myosin (205 kDa, out of range of the gel), β-galactosidase (118 kDa), BSA (69 kDa), carbonic anhydrase (42.5 kDa), soybean trypsin inhibitor (31.7 kDa), lysozyme (16.9 kDa), aprotinin (7.2 kDa, out of range of the gel).



below (**Fig. 2.8**). Once again this points to extensive decomposition of subunits, without preference for any of the isoforms.

2.2.3 Single Molecule On-Capillary Assays

The instrument used in all single molecule electrophoretic assays was an in-house built laser induced fluorescence capillary electrophoresis (LIF-CE) instrument utilizing a sheath-flow cuvette. The laser used was an Ar^+ Innova 99 (Coherent, Palo Alto, CA) at 457.9 nm. The incident laser power was 5 mW. Light was collected at 90° and passed through a 580dF40 band pass filter before being focused on a PMT (Hamamatsu, Osaka, Japan) biased at 1.2 kV. The details of the instrument have been previously published[25-28].

The starting conditions for the on-capillary single enzyme molecule assays have been adapted from those previously described for successful single molecule assays of calf intestinal alkaline phosphatase[20]. Briefly, a highly dilute enzyme solution in sample buffer (91 mM borate/9% DMSO, pH 9.5) containing 1 mM AttoPhos™ was injected onto a 10 µm i.d./150 µm o.d., 70 cm long fused silica capillary (Polymicro Technologies) for 300 s at 400 V/cm, at room temperature. The enzyme-substrate solution was incubated on capillary for 60 min. at room temperature, and at the end of incubation, the contents of the capillary were swept past the detector at 400 V/cm. The total time for an assay was on the order of 90 min. In contrast, typical incubation times for calf intestinal alkaline phosphatase were between 10-20 min., resulting in runs about 40-50 min. long.

2.2.3.1 Results of assays and the appearance of a typical electropherogram

A typical electropherogram of an on-capillary single enzyme molecule assay is shown in **Fig. 2.9 a, b**. The first figure represents the full run electropherogram.

Figure 2.8 2D-gel electrophoresis of *E. coli* alkaline phosphatase Preparation 2. First dimension standards were run in parallel to the sample on a separate gel and are not present in the 2nd dimension. The 2nd dimension (SDS-PAGE) standards are the same as in **Fig. 2.6**.

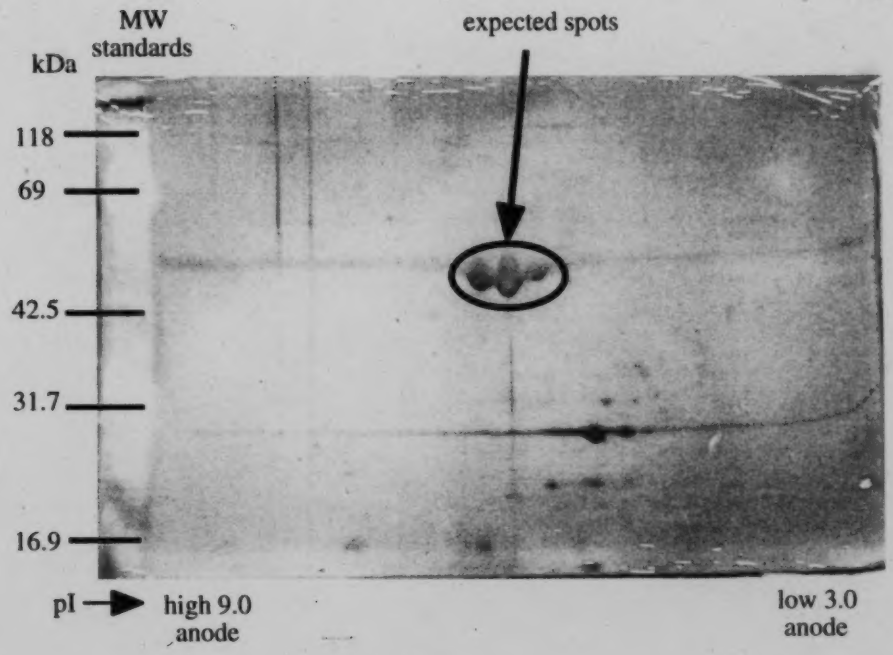
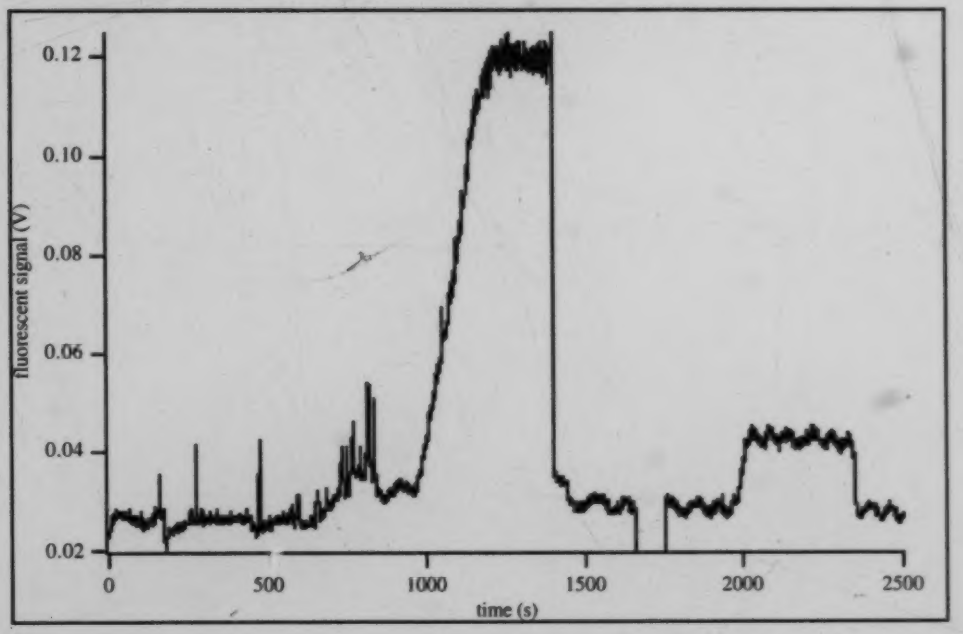
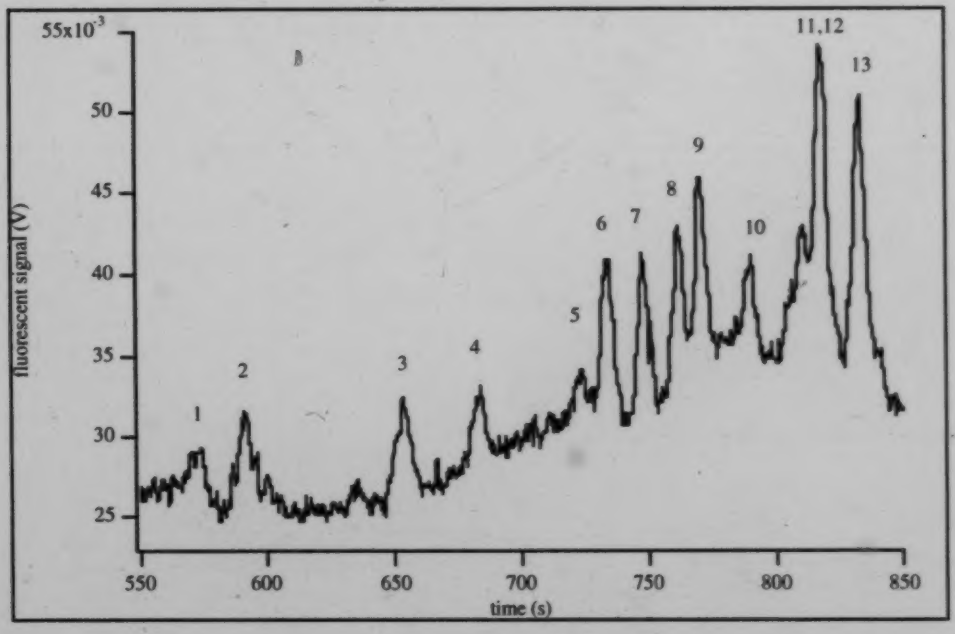


Figure 2.9 **a)** An electropherogram of an on-capillary single molecule assay using a $1/10^{10}$ dilution (approx. 10^{-15} M) of Preparation 2 *E. coli* alkaline phosphatase in sample buffer (91 mM borate, 9% DMSO, pH 9.5); **b)** product region.

a)



b)



There are three easily distinguishable general regions of interest in the single molecule assays electropherograms (times in brackets refer to this particular picture):

- product plateau (approx. 550-850 s) - there are two components to the product plateau: the plateau due to self-hydrolysis of substrate to product, and the product peaks due to enzymatic hydrolysis of substrate to product. The number of peaks will depend on the number of injected enzyme molecules within the detection volume. Overlapping peaks, and poorly resolved peaks can present a problem in the analysis of the electropherogram and may need to be discarded. In this particular experiment, a minimum of 13 enzyme molecules were present in the detection volume. Some peaks, notably 11 and 12, are poorly resolved;

- substrate plateau (approx. 980-1400 s) - the substrate migrates after the product and is visible, despite its lower fluorescence quantum yield, because of its relatively high concentration (1 mM compared with 1×10^{-9} M for the standards and enzyme product peaks). The product and substrate plateaus do not resolve in purely aqueous borate under the conditions employed in these experiments (5 min. injection and 70 cm capillary). Manipulating EOF by the addition of an organic modifier (DMSO) allows for a full separation.

- unknown plateau (approx. 2000-2350 s) - the identity of this plateau is not known. Attempts were made at discovering its identity by electrospray ionization mass spectrometry, albeit unsuccessful. However, since it has never been seen to interfere with the rest of the electropherogram or with enzyme assays, it will not be of concern.

The heights of the product peaks were fitted to a standard curve generated at the beginning of the day using at least three concentrations of Fluorescent Emitter, the product of hydrolysis of AttoPhos™. Similarly to IEF studies, when single molecule on-capillary assays were performed on Preparation 2, a wide distribution in enzyme activities was observed, evident from varying peak heights as seen in the electropherogram (see **Fig. 2.9 b**).

2.3 IMPROVEMENTS TO THE SINGLE MOLECULE ASSAYS BY CAPILLARY ELECTROPHORESIS

In order to study the structure-function relationship of this enzyme, major improvements had to be made to the assay procedure. Assay conditions had to be manipulated in such fashion so as to allow for sensitive assays in a reasonable amount of time. Furthermore, a purification method had to be developed, which would separate the three "native" isoforms from all the other fragments. To tie the activities of single molecules to their structures, structural homogeneity had to first be achieved.

2.3.1 Ultra Trace-Level Enzyme Purification

The last step in the commercial purification of Preparation 1 and Preparation 2 *E. coli* alkaline phosphatase is affinity chromatography. Due to trade secrets, the actual conditions of the purification were not revealed by Sigma's technical support staff. It appeared however, that all the more traditional approaches to protein purification had been exhausted. In view of that, and the fact that these enzyme preparations were easily fractionated by IEF, this technique was chosen to prepare structurally pure isoforms. IEF gels are normally non-denaturing and so there was a good chance of recovering active enzyme following separation. One major obstacle in the way of this was the development protocol. Most stain-based protocols use a fixing step at the beginning. This step is meant to prevent the diffusion of proteins out of the gel, which is especially problematic in low % acrylamide gels, such as IEF gels. Usually this step involves the use of strong acids, such as 20% TCA, which will invariably denature the enzyme. Even with the highly acid-resistant enzyme, such as the bacterial alkaline phosphatase used[29], I observed a full loss of activity upon a very brief fixing step with 20% TCA.

There are a number of staining protocols that preserve the activities of biomolecules. These include copper-[30] and zinc-based[31-33] stains; they also have

the advantage of being commercially available. I attempted using a zinc staining kit (BioRad) to develop IEF gels after alkaline phosphatase focusing. This is a reverse-staining technique in which the areas of the gel not containing proteins are stained while the protein bands are left transparent and can be later visualized against a dark background. Unfortunately, the resolution afforded by the zinc stain was inadequate to distinguish between the isoforms on an IEF gel.

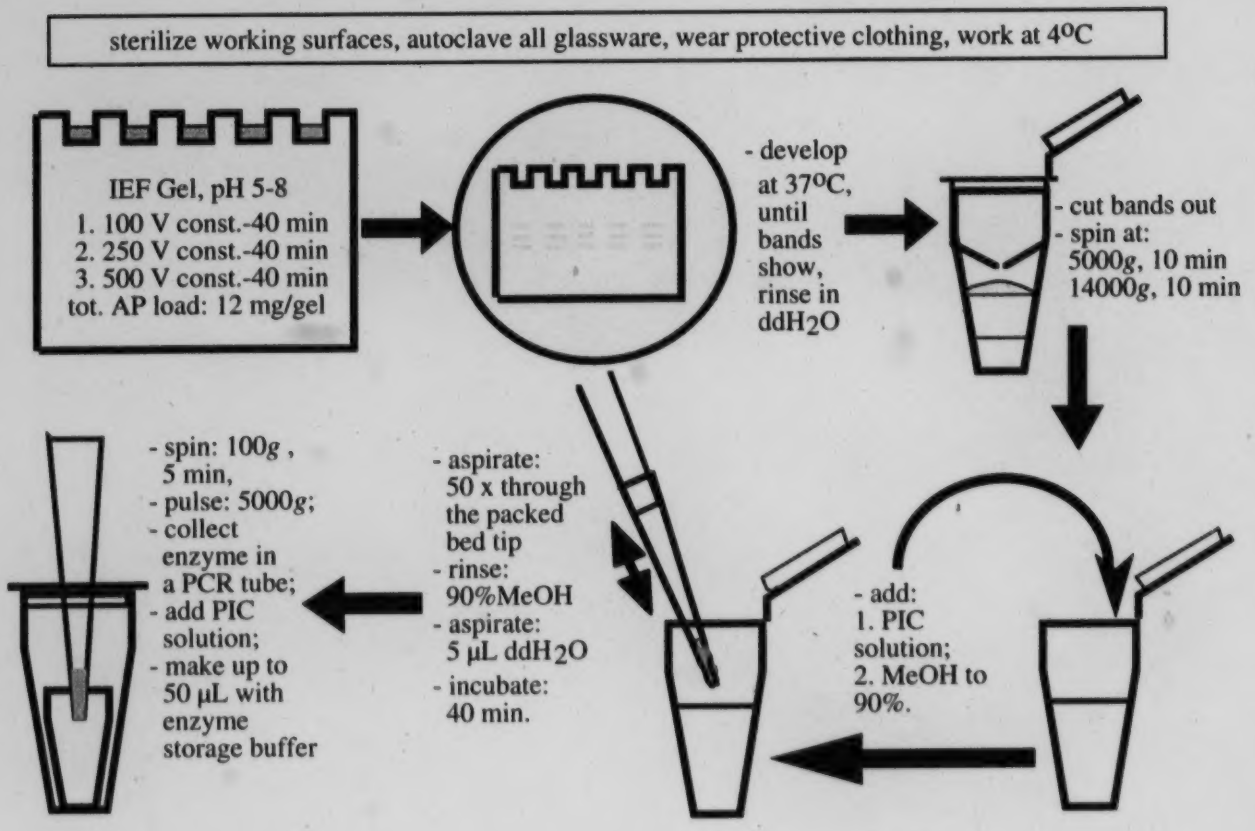
Finally, a new development method was arrived at, which was not only extremely sensitive but also had the advantage of "staining" only the active forms of the enzyme. It involves the use of AttoPhos™, the highly sensitive, fluorogenic substrate used in the on-capillary assays, in a solution that will support high activity of alkaline phosphatase. Since the method works very fast, the enzymatic bands appearing in a matter of minutes, it also does not require the fixing step. Below I describe the focusing, development and recovery of active enzyme from IEF gels. The schematic diagram of this procedure is presented in **Fig. 2.10**.

2.3.1.1 Purification Protocol for *E. coli* Alkaline Phosphatase Isozymes:

1. Work in a coldroom; clean all working surfaces with 70% EtOH; autoclave all glassware, plastic tubes, buffers and filtered ddH2O; wear gloves, labcoat, a cap and a face mask; all solutions are ice cold unless stated otherwise. These precautions ensure minimal contamination of the sample while maintaining activity.

2. Set up the Bio-Rad MiniProtean II electrophoresis cell with 2 IEF gels (Bio-Rad Ready Gels, IEF pH range 5-8). Anode buffer is 140 mL of 10x dilution of Bio-Rad 10X IEF Anode Buffer (70 mM phosphoric acid), cathode buffer is 210 mL of 10X dilution of Bio-Rad 10X Cathode Buffer (200 mM arginine, 200 mM lysine).

Figure 2.10 A schematic diagram of ultra-trace level purification of *E. coli* alkaline phosphatase isozymes


sterilize working surfaces, autoclave all glassware, wear protective clothing, work at 4°C
IEF Gel, pH 5-8
1. 100 V const.-40 min
2. 250 V const.-40 min
3. 500 V const.-40 min
tot. AP load: 12 mg/gel
- develop at 37°C, until bands show, rinse in ddH2O
- cut bands out
- spin at: 5000g, 10 min 14000g, 10 min
- add: 1. PIC solution; 2. MeOH to 90%.
- aspirate: 50 x through the packed bed tip
- rinse: 90%MeOH
- aspirate: 5 µL ddH2O
- incubate: 40 min.
- spin: 100g , 5 min,
- pulse: 5000g;
- collect enzyme in a PCR tube;
- add PIC solution;
- make up to 50 µL with enzyme storage buffer

3. Prepare a 1/40 dilution of Sigma *E. coli* alkaline phosphatase (Preparation 2, see **Section 2.2**) in Bio-Rad sample buffer (50% glycerol). The high viscosity sample buffer ensures that the solution will sink to the bottom of the well. Load approximately 10 µL/well x 8 wells/gel (omit the outermost lanes) using a fresh gel loading tip for each well. Remove all bubbles.
4. Run a 3-step program, all steps at constant voltage:

   - 100 V for 40 min.

   - 250 V for 40 min.

   - 500 V for 15 min.

   This program does not use high voltages and is relatively short which helps minimize enzyme unfolding.
5. When finished, place both gels in 50 mL each imidazole solution which is 10X dilution of Bio-Rad 10X Zinc Stain Solution A (BioRad, Hercules, CA). The imidazole solution appears to slow down the diffusion of enzyme after electric field is removed. Shake on an orbital shaker for 10 min. at 50 rpm, switch to ddH_2O for 5 min. and then to development buffer which is approximately 1 mM AttoPhos™ (JBL Scientific, San Luis Obispo, CA) in 1 M Tris, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, pH 8.0 at 37°C and shake until yellow bands appear. This development buffer uses Tris, which is a known activator of *E. coli* alkaline phosphatase[1]. In addition, the pH of 8.0 is the optimum pH for this enzyme. Switch to ddH_2O for approximately 1 min. to rinse the gel.
6. Process one gel at a time: remove the gel from water and place in a sterile Petri dish. Using square microscope cover slips which had been soaked in 0.01 M HCl overnight and rinsed in autoclaved ddH_2O prior to use, cut out the bands using a fresh slip for each band. The slips have a thin edge and are inexpensive. Using a pipettor with 1000 µL pipette tips and taking care not to cross-contaminate samples use a little bit of suction to place each band in an Amicon Ultrafree DA device

(Millipore, Bedford, MA). The bands are numbered 1-5 starting with the cathodic end. Band #5 is very diffuse and barely visible. Gels are numbered A & B starting with the first gel processed.

7. When all bands are cut out, spin the tubes at 5000g for 10 min., then at 14000g for 10 min. The initial spin causes the gel to nebulize and stop at the filtration membrane. It was observed, that when high g-force spin was performed first, the membrane often broke under the pressure of the gel. To each tube add 5 µL of Roche Complete EDTA-free Protease Inhibitor Cocktail (Roche, cat.# 1873580) prepared by dissolving one tablet in 300 µL ddH2O. The protease inhibitors stop the enzyme from being digested by any contaminating proteases. Pool A & B tubes for each band together (i.e. #1A+ #1B, #2A + # 2B, etc.).
8. Take 20 µL of sample from each tube and add 180 µL HPLC-grade MeOH. Using 200 µL ProTips (Amika Corp., cat.# PT050) aspirate each sample 50 times (100 passages through the packing). In the presence of high organic solvent content the enzyme will bind to the hydrophobic packing material. Rinse with 20 µL MeOH and aspirate 5 µL 0.5 mM $MgCl_2$, 0.5 mM $ZnCl_2$ into the tip. The aqueous solution will allow for the elution of enzyme while the metal ions will prevent it from denaturing. Incubate for a minimum of 40 min. and then spin the tip inside a plastic tube to recover the sample (5 min. at 100g and a short pulse at 5000g); collect samples into previously passivated and autoclaved PCR tubes. Alternatively, use sterile, siliconized tubes (Fisher, Pittsburgh, PA). Add 2 µL of the above protease inhibitor cocktail to each sample and make up to 50 µL with 50% glycerol, 0.5 mM $MgCl_2$, 0.5 mM $ZnCl_2$. Store at 4°C.

The success of the purification procedure could then be assessed by re-running the bands on a new IEF gel along with the commercial sample. Due to the small amount of enzyme obtained during the purification, only one of the bands, the most

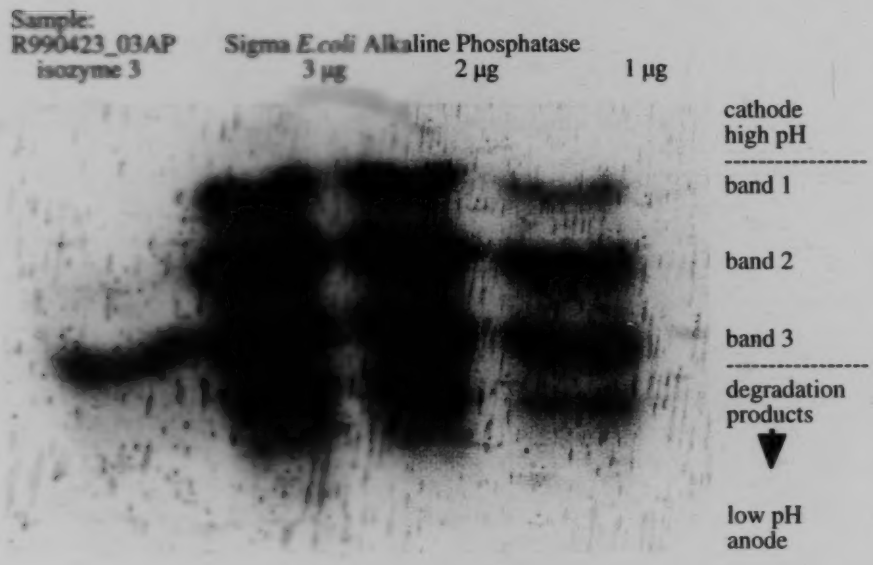
abundant band #3, was re-run. Development was again done with AttoPhos™ to ensure that the recovered isozyme was in fact active. There was very little apparent contamination in that band as can be seen in **Fig. 2.11**, and thus the purification was deemed successful in obtaining structurally pure and active isoforms of *E. coli* alkaline phosphatase.

As can be seen from the gel however, two other species appeared to be active in the commercial Preparation 2 (see **Fig. 2.11**). This supports the earlier hypothesis, that further proteolytic processing of the enzyme results in a number of other active enzyme species in addition to the "native" isoforms. Using above method for the purification of the isoforms, these additional species can be disregarded.

2.3.2 Improvements to the Assay Conditions

Aside from the purification of isoforms, the assay conditions also needed improvement. Firstly, the existing assay used borate/DMSO buffer at pH 9.5 for separation, whereas the optimum pH for *E. coli* alkaline phosphatase is around 8.0. Enzymatic activity is also stimulated by the use of the so-called transphosphorylating buffers - buffers which can readily accept the phosphate moiety after it is removed from the substrate. Amino-alcohols, including Tris, fall into this category. Therefore, attempts were made to use Tris at pH of 8.0 as the buffer. Similarly to borate alone, Tris alone was unable to resolve the product and substrate plateaus. Upon addition of DMSO, a reaction occurred between the two components, as evidenced by a strong cabbage-like smell. The resulting solution also failed to resolve the plateaus. Thus Tris was abandoned as a buffer. Another suggested buffer, the bicarbonate/carbonate buffer system also did not give adequate resolution of the plateaus. The original borate/DMSO buffer was therefore retained for further assays.

Figure 2.11 Assessment of ultra-trace level purification of *E. coli* isoforms. Isoform 3 was re-run on a BioRad IEF ready gel, along with 3 different concentrations of commercial Preparation 2, and developed with AttoPhos™ to test for the activity.

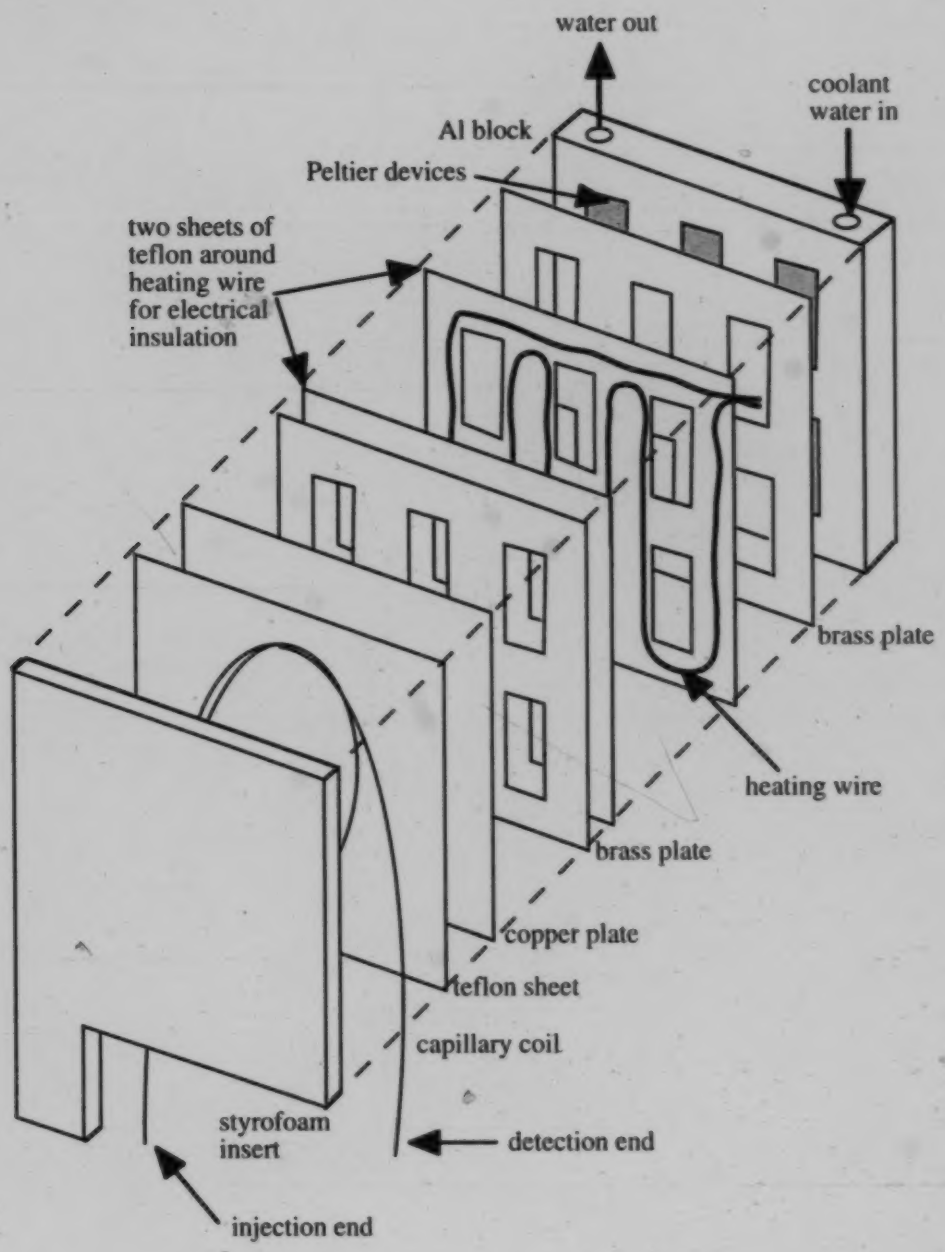


Another possibility to increase the activity of the bacterial enzyme was to raise the temperature of incubation. The temperature chosen for the incubations was 40°C. At this temperature, the activity of bacterial alkaline phosphatase increases two-fold over that at room temperature(1). This allowed for the shortening of incubation time from one to one-half hour, and therefore the total assay time was now 60 min. Since the higher temperature was maintained not only during incubation but throughout the run, buffer composition had to be manipulated to compensate for higher current and electroosmotic flow, both resulting from higher temperature. The concentration of DMSO was raised from 10% to 15% in the running buffer and from 9% to 13.5% in the sample buffer. This allowed good separation in a reasonable amount of time. Further gain in total assay time was made by using overlapping injections - the new injection was performed soon after the electroelution of the substrate plateau. This resulted in the reduction of total assay time to approximately 50 min.

2.3.2.1 Peltier Heating/Cooling Device

Heating of capillaries was traditionally done in our lab by means of AirTherm devices (World Precision Instruments, Sarasota, FL) - essentially a heating coil over which air is blown into an enclosed compartment. There were two drawbacks to using these units: first, they created an undesirable amount of capillary vibration, and second, their heating was uneven and hard to control. Therefore, a Peltier-driven heating/cooling device was designed which could encase the capillary and heat or cool it with relatively high precision of 0.1°C. These devices were designed to become an integral part of our in-house built CE systems. The basic set-up this device is shown and described in **Fig. 2.12**. In this unit, six Peltier units are connected in series and sandwiched between an aluminum block and a copper plate. Within this sandwich, an

Figure 2.12 A schematic diagram of a Peltier heater/cooler. The sandwiched assembly is housed within a Plexiglass box (not shown). For the full description, see **Section 2.3.2.1**.



additional sandwich of a thin brass plate and two layers of Teflon houses a nickel/chromium heating wire. This sandwich has holes cut out in it to fit over the Peltiers. Both sandwiches are encased in Plexiglass with only the back side of the aluminum block open to air. The block itself has channels machined within it to allow coolant flow. There are two resistive thermal devices (RTDs) placed on the top copper plate - one provides feedback to the temperature controller while the other is connected to a temperature monitor which in turn is connected to the computer where the temperature readings are acquired in LabView along with current and signal data. The RTDs are covered with yet another layer of Teflon. The capillary is threaded through small i.d. Teflon tubing and coiled on top of the Teflon sheet. A styrofoam piece is then inserted into the Plexi unit and a hinged door is tightly closed with a screw. The power to Peltiers and the heating wire is supplied by an in-house built power supply controlled by an Omega Temperature Process Controller, Model CN76060 (Omega Engineering, Stamford, CT.).

2.4 STRUCTURAL VS. FUNCTIONAL HOMOGENEITY IN *E. COLI* ALKALINE PHOSPHATASE

With the bacterial isoforms purified and assay conditions improved to allow for shorter experiments, the search for the relationship between the structures and function of the isoforms could continue. Each of the three native isoforms was assayed on capillary and compared to the commercial mixture.

2.4.1 On-capillary Single Enzyme Molecule Assays: Experimental Conditions

Starting with the stored purified isoforms (see **Section 2.3.1.1**), isoform dilutions of between $5/10^6$ and $1.25/10^3$, and dilutions of commercial Preparation 2 of between $3/10^{11}$ and $1/10^{10}$ were prepared for injection. The dilutions were made into

sample buffer (see below) containing 1 mM AttoPhos™ substrate. The run conditions are described below for all of the runs:

1) inject standard @ 400 V/cm for 2 sec. (manually controlled)

2) rinse capillary and electrode in running buffer

3) CE running buffer @ 400V/cm for 60 sec

4) inject a dilution of enzyme sample for time t_{inj} at 400 V/cm

5) rinse capillary and electrode in running buffer

6) CE running buffer for time t_{push} to move plug into the heater zone

7) incubate for time t_{inc}

8) CE running buffer

Constants:

running buffer = 85 mM borate (pH 9.5)/15% DMSO,

sample buffer = 86.5 mM borate (pH 9.5)/13.5% DMSO,

sheathflow = 100 mM borate, pH 9.5,

standard = Fluorescent Emitter at specified concentration, in sample buffer,

variables = standard concentration, sample, injection time, push time, and incubation time are given in **Table 2.1**.

2.4.2 On-capillary Single Enzyme Molecule Assays: Results

The results for sample runs representing each isoenzyme are shown in **Fig. 2.13**. The instrument was calibrated daily using the standards shown in **Table 2.1**. Peak area above the baseline was measured using PeakFit (SPSS Science, Chicago, IL). The data for each day was then fitted to corresponding daily standard curves to obtain the concentration of product, Fluorescent Emitter, and divided by the incubation time for each run to arrive at the activity of each molecule. The activity distributions of the three isoforms studied are presented in **Fig. 2.14**.

Table 2.1 Variables in on-capillary single molecule assays of purified isoforms and commercial Preparation 2. For isoform descriptions, see **Section 2.1**. Commercial Preparation 2 is designated SEC-AP. Dilution are made from stock: initially in 50% glycerol, 1 mM $ZnCl_2$, 1 mM $MgCl_2$; then at least 4 orders of magnitude in 100 mM borate, pH 9.5; finally 1/10 in running buffer. Dilutions of less than $2/10^5$ are made without the glycerol step.

Run	[Standard]	Sample	Dilution	$t_{injection}$	t_{push}	$t_{incubation}$
R990518_02	$4*10^{-9}$ M	isoform 1	$1/10^5$	300 s	136 s	1800 s
R990518_03	$4*10^{-9}$ M	isoform 1	$1/10^5$	330 s	120 s	1960 s
R990518_04	$4*10^{-9}$ M	isoform 1	$2/10^5$	360 s	120 s	1800 s
R990518_05	$2*10^{-9}$ M	isoform 1	$2/10^5$	360 s	190 s	1800 s
R990518_06	$2*10^{-9}$ M	isoform 2	$5/10^6$	360 s	120 s	1800 s
R990518_07	$1*10^{-9}$ M	isoform 2	$5/10^6$	360 s	120 s	1800 s
R990518_08	$1*10^{-9}$ M	isoform 2	$1/10^5$	360 s	120 s	1800 s
R990518_09	$8*10^{-10}$ M	isoform 2	$4/10^5$	380 s	120 s	1800 s
R990519_02	$1*10^{-9}$ M	isoform 2	$5/10^5$	360 s	120 s	1800 s
R990519_03	$2*10^{-9}$ M	isoform 2	$1/10^4$	360 s	120 s	1800 s
R990519_04	$1*10^{-9}$ M	isoform 2	$1/10^4$	360 s	120 s	1860 s
R990519_05	$2*10^{-9}$ M	isoform 2	$1.25/10^3$	360 s	120 s	600 s
R990519_06	$2*10^{-9}$ M	isoform 2	$1/10^4$	360 s	120 s	600 s
R990519_07	$1*10^{-9}$ M	isoform 2	$1/10^5$	360 s	120 s	600 s
R990519_08	$2*10^{-9}$ M	isoform 3	$1/10^5$	390 s	120 s	1800 s
R990519_09	$1*10^{-9}$ M	isoform 3	$1/10^5$	370 s	120 s	1800 s
R990519_10	$2*10^{-9}$ M	isoform 3	$2/10^5$	370 s	120 s	1800 s
R990520_03	$2*10^{-9}$M	isoform 3	$2/10^5$	380 s	120 s	1800 s
R990520_04	$1*10^{-9}$M	isoform 3	$1/10^5$	360 s	120 s	1800 s
R990520_05	$2*10^{-9}$M	SEC-AP	$1/10^{10}$	360 s	120 s	1800 s
R990520_06	$3*10^{-9}$M	SEC-AP	$5/10^{11}$	390 s	120 s	1920 s
R990520_07	$2*10^{-9}$M	SEC-AP	$5/10^{11}$	360 s	120 s	1800 s
R990520_08	$1*10^{-9}$M	SEC-AP	$4/10^{11}$	360 s	120 s	1800 s
R990521_02	$2*10^{-9}$M	SEC-AP	$3/10^{11}$	360 s	170 s	1800 s
R990521_03	$1*10^{-9}$M	SEC-AP	$3/10^{11}$	365 s	120 s	1800 s

Figure 2.13 Electropherograms of highly purified *E.* coli alkaline phosphatase isoforms: **a)** isoform 1, **b)** isoform 2, and **c)** isoform 3. The vertical and horizontal scales are the same for all figures.

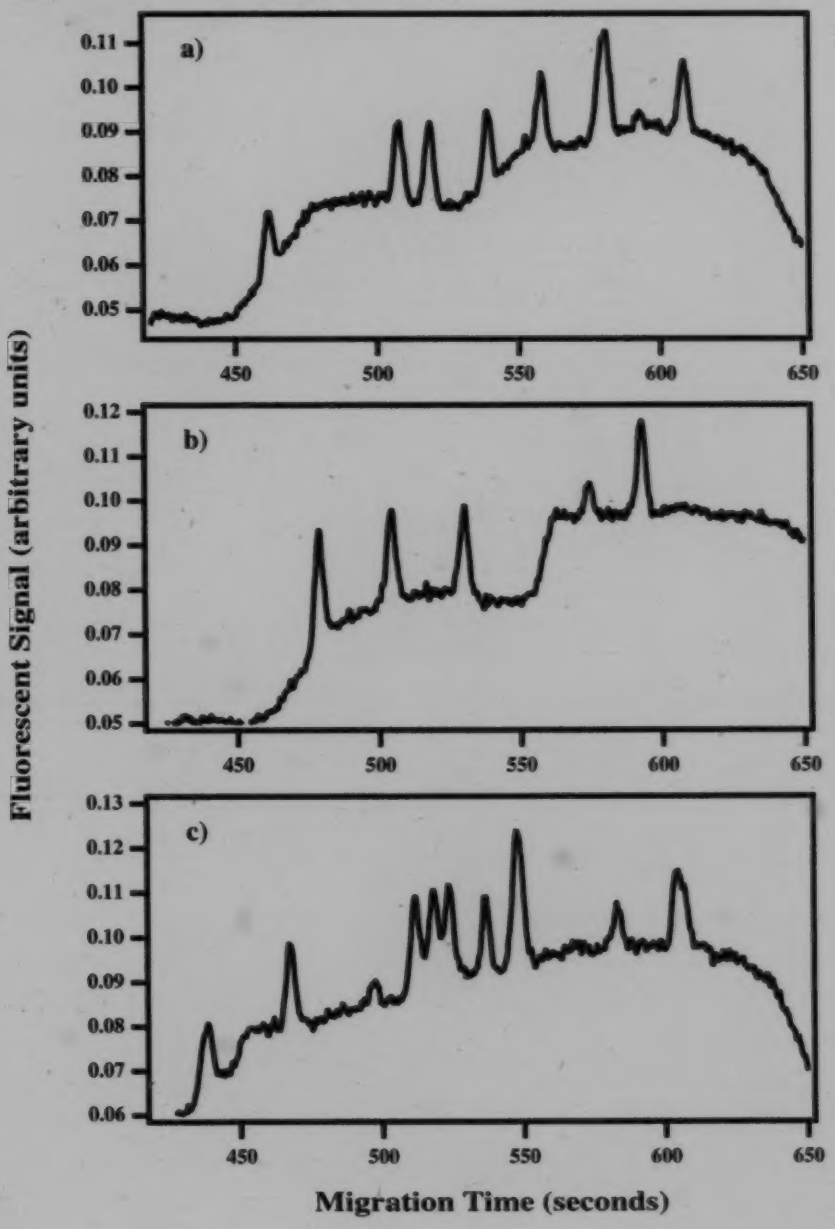
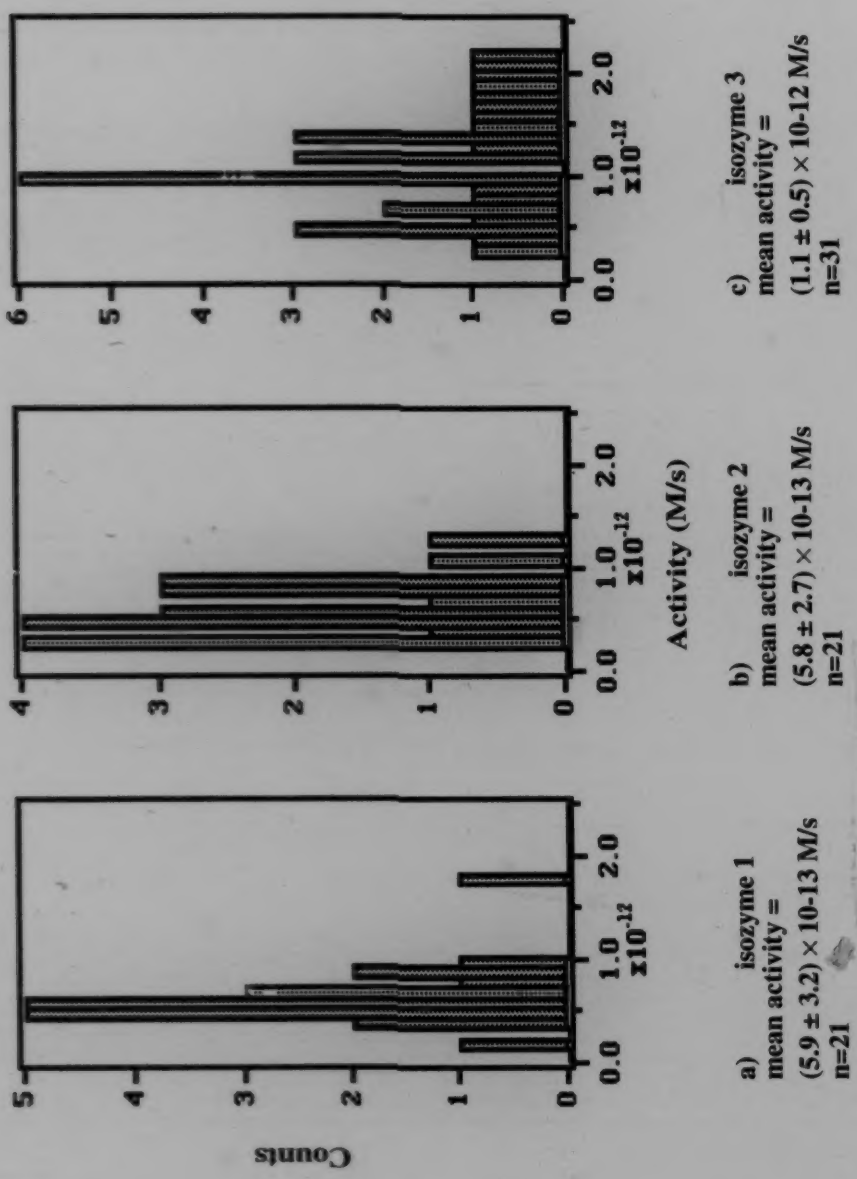


Figure 2.14 Activity distribution for three highly purified *E. coli* alkaline phosphatase isoforms: **a)** isoform 1, **b)** isoform 2, and **c)** isoform 3. Binning is arbitrary, bin size is 0.1×10^{-12} M/s.



2.4.3 On-capillary Single Enzyme Molecule Assays: Discussion

As can be seen from the data, the activities of individual molecules of each isoform sample were homogeneous to within 50%. This was also true for isoform-to-isoform comparison. This leads to two conclusions: first is that activities of all molecules of individual, highly purified, and hence structurally homogeneous, isoforms are identical within experimental error; second is that activities of *E. coli* alkaline phosphatase isoforms do not differ significantly from one another. The first conclusion seems to agree with common sense: molecules that are structurally identical should behave identically assuming that the structures are stable on the time frame of the experiment. The second conclusion may only hold true for this particular enzyme. Since the difference between the "native" isoforms is very small, a single amino acid deletion at the end of a chain 450 amino acids in length, one would not expect to see much of a difference between the activities of individual isoforms. This is especially unlikely when the location of the deletion is compared to the location of the active site and the overall physical shape of the molecule. The active site in *E. coli* alkaline phosphatase is located at position 103 (or 102, depending on the isoform, see **Fig. 2.2**) and deep within the molecule, facing away from the twenty-odd amino acid chain protruding from an otherwise globular molecule, at the end of which the deleted Arg resides (**Fig. 2.1**). It is easy to appreciate that a removal of the terminal amino acid should not affect the activity of the entire enzyme. It then seems plausible, that further deletions as speculated in **Section 2.2.1**, would produce a number of active species whose activity may start to change as the digest progresses, getting closer to the first turn which occurs at the amino acid 17/16 (Ile) in the processed enzyme. This further digestion would certainly be capable of inducing changes in the activity by disrupting the folding of the molecule. Eventually the activity is lost when the enzyme unfolds beyond repair.

Yeung has suggested that the heterogeneity in enzymatic activities of lactate dehydrogenase that his group observed by single molecule enzyme assays is due to conformational changes in the secondary structure of the enzyme molecules; these changes must be stable within the timeframe of his experiments[34](see **Section 1.2.3**). In contrast our group has previously argued that this type of heterogeneity would more likely be due to differences in primary structures of isoforms or isozymes. In view of the above results, I feel that our explanation is more plausible, and that there is no need to invoke the energy landscape model[35], where molecules of a given enzyme constantly switch between different, yet stable arrangements. The energy landscape model would be better suited to explain extremely short-lived activity fluctuations in individual enzyme molecules as they continuously unfold and refold. This has indeed been observed by Sunney Xie and his group at the Pacific Northwest National Laboratory [36]. It is unlikely that any of these secondary-level arrangements would persist on the timescales of our, or Yeung's experiments; it is more likely that they would be averaged over such timescales. In contrast, the differences in primary structures are stable, and much more likely to affect the activities of individual molecules. To this end we have postulated that what Yeung's group observed was in fact due to structural heterogeneity of the lactate dehydrogenase preparation in question. To test this, a sample of lactate dehydrogenase was obtained from the same source as that indicated by Yeung[34]. When subjected to IEF, the enzyme separated into a number of bands, suggesting structural heterogeneity.

2.5 THERMAL DENATURATION AND THE DEATH OF *E. COLI* ALKALINE PHOSPHATASE MOLECULES

In this study, dilute bulk samples of *E. coli* alkaline phosphatase were subjected to increasingly longer incubations at 96°C before being injected onto the capillary for single molecule assays of enzymatic activity. The number of surviving molecules as

well as their activities were monitored in order to establish the pathway by which this enzyme denatures and dies.

2.5.1 Denaturation Protocol and Assay Conditions

The enzyme used in denaturation studies was the commercial Preparation 2 (see **Section 2.2**). Prior to heating, the enzyme was diluted $1/10^6$ in 50% glycerol. Aliquots of 4 µL were placed in 600 µL plastic tubes and heated at 96°C for a time between 30 and 900 seconds. At the end of that time, 396 µL of ice-cold borate (100 mM, pH 9.5) were added to the enzyme sample. The sample was immediately diluted another 50- or 100-fold and injected on to the capillary. Alternatively, if immediate dilution was impossible, the sample was stored on ice until ready for injection. The dilutions were made into sample buffer containing 1 mM AttoPhos™ substrate. The capillary was kept at 40°C throughout the experiments. The CE protocol is as follows:

1) inject standard @ 80 V/cm for 10 sec. (manually controlled)

2) rinse capillary and electrode in running buffer

3) electrophorese running buffer @ 400 V/cm for 60 sec.

4) inject a dilution of enzyme sample @ 400 V/cm for a time t_{inj}

5) rinse capillary and electrode in running buffer

6) CE running buffer for 120 sec. to move the enzyme plug into the heater zone

7) incubate for time t_{inc} at 40°C

8) electrophorese running buffer at 400 V/cm

All buffe re the same as described in **Section 2.4.1**. The variables (standard concentration, injection time, dilution factor, incubation time, and heating time) are given in **Table 2.2**.

Table 2.2 Run conditions for the thermal denaturation study of *E. coli* alkaline phosphatase.

Run	t_{heat} (s)	[FE] (M)	t_{inj} (s)	dilution	t_{inc} (s)
R991004_02	0	$3 * 10^{-9}$	270	$5/10^{11}$	1800
R991004_03	0	$4 * 10^{-9}$	268	$1/10^{10}$	1800
R991004_04	0	$2 * 10^{-9}$	270	$1/10^{10}$	1800
R991005_02	0	$2 * 10^{-9}$	285	$1/10^{10}$	1800
R991005_03	0	$3 * 10^{-9}$	290	$1/10^{10}$	1800
R991005_04	0	$3 * 10^{-9}$	300	$1/10^{10}$	1825
R991005_05	30	$2 * 10^{-9}$	300	$1/10^{10}$	1800
R991005_06	60	$4 * 10^{-9}$	300	$1/10^{10}$	1800
R991006_02	0	$2 * 10^{-9}$	300	$1/10^{10}$	1800
R991006_03	0	$3 * 10^{-9}$	300	$2/10^{10}$	1800
R991006_04	60	$1 * 10^{-9}$	300	$2/10^{10}$	3300
R991006_05	120	$1 * 10^{-9}$	330	$2/10^{10}$	1800
R991006_06	180	$2 * 10^{-9}$	330	$2/10^{10}$	1800
R991006_07	240	$3 * 10^{-9}$	330	$2/10^{10}$	1800
R991007_02	0	$1 * 10^{-9}$	266	$2/10^{10}$	1800
R991007_03	255	$2 * 10^{-9}$	330	$2/10^{10}$	1800
R991007_04	300	$3 * 10^{-9}$	330	$2/10^{10}$	1800
R991012_02	0	$1 * 10^{-9}$	330	$2/10^{10}$	1800
R991012_03	360	$2 * 10^{-9}$	330	$2/10^{10}$	1800
R991012_04	420	$3 * 10^{-9}$	330	$2/10^{10}$	1800
R991012_05	480	$1 * 10^{-9}$	330	$2/10^{10}$	1800
R991012_07	540	$3 * 10^{-9}$	405	$2/10^{10}$	1800
R991013_02	0	$1 * 10^{-9}$	340	$2/10^{10}$	1800
R991013_03	600	$2 * 10^{-9}$	330	$2/10^{10}$	2055
R991013_04	660	$3 * 10^{-9}$	332	$2/10^{10}$	1800
R991013_05	720	$4 * 10^{-9}$	330	$2/10^{10}$	1800
R991013_06	780	$5 * 10^{-9}$	330	$2/10^{10}$	1800
R991014_02	0	$1 * 10^{-9}$	330	$2/10^{10}$	1800
R991014_03	840	$2 * 10^{-9}$	495	$2/10^{10}$	1860
R991014_04	900	$3 * 10^{-9}$	330	$2/10^{10}$	1800

2.5.2 Results of the Thermal Denaturation Study

Both the number of surviving alkaline phosphatase molecules and their activities were measured in this study. The number of counted molecules is given in **Table 2.3** along with correction factors that take into account varying lengths of injection and dilutions of stock enzyme. An injection time of 330 seconds and a dilution of $2/10^{10}$ were chosen as standard. Except for $t_{heat} = 0$ s and 60 s, all experiments were done only once. For these experiments the standard deviation in the number of counted molecules, governed by Poisson statistics, is given by the square root of that number. This is then propagated through the correction factors. In the case of $t_{heat} = 0$ s, where 12 assays were done, the mean and its standard deviation are reported. For $t_{heat} = 60$ s (2 runs), the average of the two runs is reported with standard deviation obtained by applying Poisson statistics to the two individual counts and propagating the error through the correction factors. The plot of the corrected number of surviving molecules as a function of heating time is shown in **Figure 2.15**. An exponential decay curve is fitted to the data, with a characteristic time constant of 500 s.

The average activities of the surviving molecules are shown in **Table 2.4** along with their standard deviations. The plot of these activities as a function of heating time is shown in **Figure 2.16**. Once again, an exponential decay curve fits the data, having a characteristic time constant of 176 s. The data can also be fitted with a linear plot although the R^2 value is a relatively low 0.5465.

2.5.3 Thermal denaturation and the death of enzyme molecules: discussion

The results of this study have to be interpreted carefully. There is clearly a decrease in both the number of surviving molecules and their average activities with heating time. These decreases are not equal however - whereas the number of

Table 2.3 Results of thermal denaturation study - number of surviving molecules according to the length of denaturation step. For the t_{heat} = 0 s and 60 s, the corrected number of peaks and the standard deviation are obtained from a number of individual experiments and therefore they represent the average and its standard deviation of all the runs. For all other t_{heat} the experiments were done only once; the standard deviation is governed by Poisson statistics.

Heat	Counted Peaks		Correction Factors		Corrected Peaks	
time (s)	#	StdDev	t_{inj}	dilution	#	StdDev
0	127	11	variable	variable	18.3	6.4
30	10	3.2	1.10	2	22	7
60	19	4.3	variable	variable	14	1
120	13	3.6	1.00	1	13	4
180*	11	3.3	1.00	1	11	3
240	8	2.8	1.00	1	8	3
255	15	3.9	1.00	1	15	4
300	8	2.8	1.00	1	8	3
360	11	3.3	1.00	1	11	3
420	6	2.4	1.00	1	6	2
480	1	1	1.00	1	1	1
540	5	2.2	0.81	1	4	2
600	1	1	1.00	1	1	1
660	1	1	0.99	1	1	1
720	1	1	1.00	1	1	1
780	4	2	1.00	1	4	2
840	7	2.6	0.67	1	5	2
900	1	1	1.00	1	1	1

Figure 2.15 Thermal denaturation of *E. coli* alkaline phosphatase. The data was fitted with an exponential curve. The equation is:
$y = (-2.6 \pm 5.8) + (21.6 \pm 5.2)\, e^{(-0.0020 \pm 0.0011)x}$, $\chi^2 = 137.2$

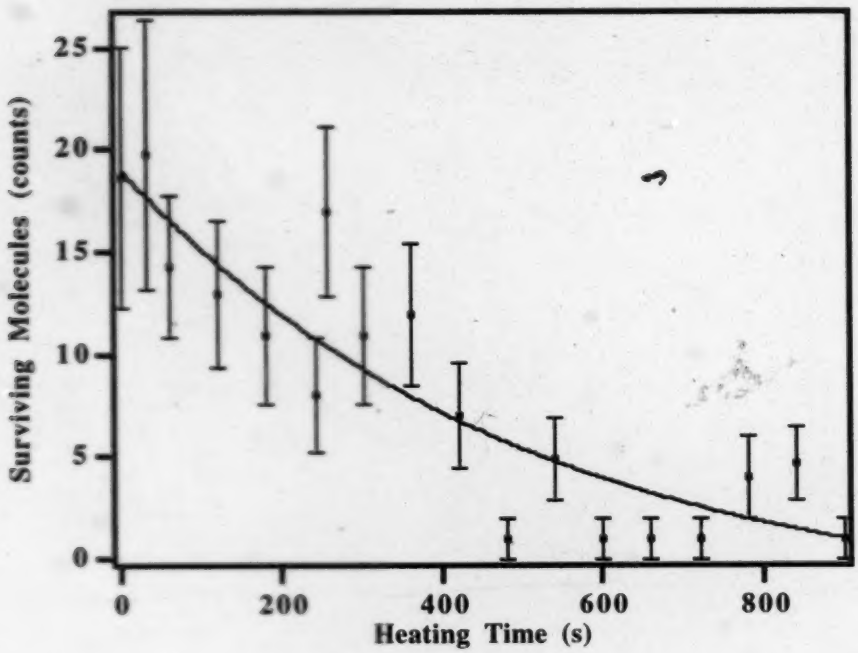


Table 2.4 Results of thermal denaturation study - average activities of remaining molecules

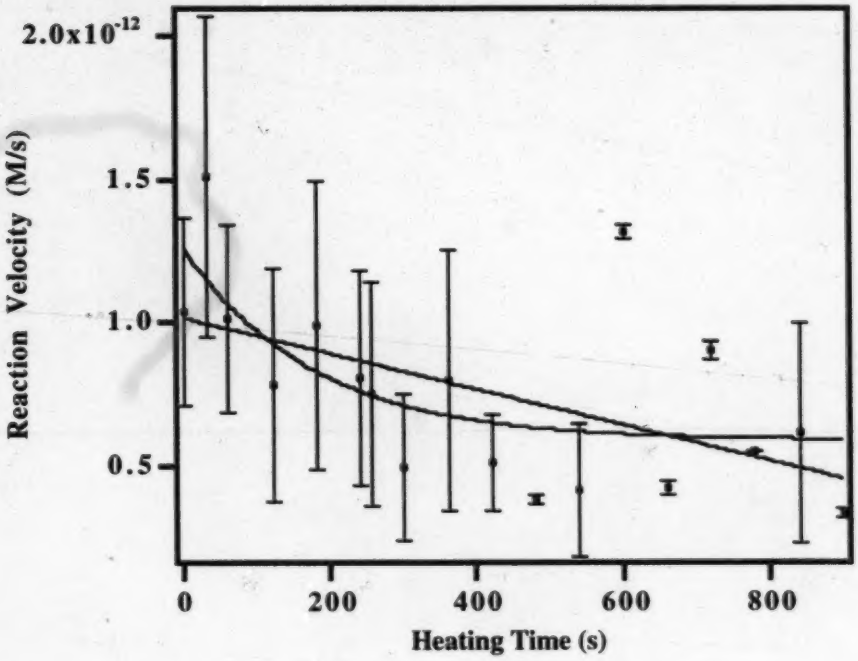
Heating time (s)	Activity ($\times 10^{-12}$ M/s)	
	Average	Error
0	1.04	0.32
30	1.51	0.56
60	1.01	0.33
120	0.79	0.41
180	0.99	0.50
240	0.81	0.38
255	0.75	0.39
300	0.50	0.25
360	0.80	0.46
420	0.51	0.16
480	0.385	0.018
540	0.42	0.23
600	1.318	0.024
660	0.422	0.023
720	0.907	0.029
780	0.5540	0.0011
840	0.61	0.39
900	0.338	0.019

Figure 2.16 Average activity of surviving *E. coli* alkaline phosphatase molecules during a thermal denaturation experiment. The data was fitted with a linear and an exponential curve. The equations are:

linear: $y = (-6.2 \pm 2.4) \times 10^{-16}\, x + (1.02 \pm 0.12)$, $R^2 = -0.5465$

exponential: $y = (5.9 \pm 1.2) \times 10^{-13} + (6.7 \pm 2.1) \times 10^{-13}\, e^{(-0.0057 \pm 0.0045)x}$,

$\chi^2 = 1.10 \times 10^{-24}$



survivors decreases 20-fold between 0 and 500 seconds of heating time, their average activity drops only by a factor of 2 over the entire 900 seconds of heating.

In a Cheshire Cat model one would expect the number of survivors to stay approximately the same as the heating times got longer. Their average activities would see a drop with increasing denaturation times however, as a result of the enzyme undergoing increasingly dramatic conformational changes. This does not appear to be the case here as the number of survivors decreases more drastically than their average activities.

In the catastrophic model of enzyme denaturation the number of survivors would decrease with the heating time while their activities should remain constant. This is almost evident in this experiment. Although the average activities of surviving molecules decrease somewhat with increasing heating times, this decrease is far smaller than that in the number of survivors. In addition, the experiment has an inherent flaw in it, which may further explain that decrease. Thermal denaturation of *E. coli* alkaline phosphatase, in the absence of additional denaturants (DTT, EDTA), is a reversible process; renaturation has been observed in heat inactivated samples which had been cooled prior to assays[15]. Since the average incubation time on capillary is 30 min., this may give enough time for some denatured enzyme molecules to begin the process of renaturation. Since those molecules that do renature throughout the incubation period have less time to process the substrate, they would clearly result in peaks which are smaller than those produced by enzyme molecules which never lost their activities. Obviously, with longer denaturation times, the proportion of molecules undergoing the renaturation process would increase, resulting in a decreasing average activity values. In addition, this would lead to an overestimation of the number of survivors. There is no easy way to correct for that problem in our experimental set-up. Including denaturing agents in one of the solutions would probably help in the full inactivation of the enzyme. Their addition however, would introduce another variable in the

experiment - the inactivation would no longer be due to heat alone. Furthermore, adding denaturants may cause some molecules not undergoing the heat inactivation to lose their activity. To overcome this likely effect of prolonged incubation times, the incubation itself would have to be eliminated. For that, the experimental set-up would need a full overhaul, an issue which is beyond the scope of this work, but will be dealt with to a limited extent in the Future Work section.

In addition to confirming the catastrophic model of heat denaturation and death for *E. coli* alkaline phosphatase the experiments brought another interesting point to my attention. In both of the denaturation plots (survivors vs. time, **Figure 2.15**, and activity vs. time, **Figure 2.16**) there is a clear increase in both the number of survivors and their average activities after the first 30 seconds of heating. This suggest a possible heat activation process whereby the enzyme becomes more active after a very short time spent at a high temperature. This would agree well with the activation of *E. coli* alkaline phosphatase observed by Garen and Levinthal[1] with temperature increasing all the way up to 80°C.

2.6 CONCLUSIONS

Successful studies on single molecules of *E. coli* alkaline phosphatase were possible only after some improvements to the set-up used previously for single molecule studies on calf intestinal alkaline phosphatase. Introducing a capillary temperature controller allowed for the assays to be performed at temperatures closer to the optimum for this enzyme. This in turn made possible the observation of differences in the activities of the single molecules. The heterogeneity of this enzyme's commercial preparations proved to be the result of not the differences in the enzyme's individual isoforms' structures, but rather the result of the degradation of the enzyme in those preparations. Further evidence for this degradation was provided by gel electrophoresis of the commercial and purified samples. When the isoforms were

purified to high homogeneity, the differences all but disappeared, to within the experimental error, for the molecules of the same isoform. The differences between molecules of the different isoforms also became very small. This is not surprising when the structures of the three isoforms are taken into consideration. The minute differences between the three "native" isoforms should not produce the large differences in the activities of the molecules in bulk samples, previously observed. Nevertheless, it would be rash to assume that the isoforms must be identical in their reaction rates. After all, their structures, although very similar, are not identical. The evidence shows that there very well may be small differences in activities of the isoforms - these differences, however are not nearly as large as previously seen.

Thermal denaturation of *E. coli* alkaline phosphatase was shown to be a catastrophic process. The conclusion was drawn carefully, because the experiment had a possible flaw. The evidence still pointed towards a catastrophic death as opposed to a gradual unfolding of the enzyme with associated gradual decreases in activities of individual molecules, the Cheshire Cat model. In addition, activation of the enzyme upon very short heating at high temperature has been observed, in accordance with the high thermal stability of this particular alkaline phosphatase and some previous studies showing increasing reaction rates with increasing temperatures, all the way to 80°C.

The single molecule studies on this enzyme helped to show that there is a correlation between the enzyme's primary structure and its function (activity). This correlation appears to be stronger than that proposed for by Yeung *et al.*, who suggested that activity differences between various isoforms are due to secondary structure fluctuations. It is important to point out, that these conclusions could only be reached through studies of single enzyme molecules.

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3

Studies on Single Molecules of Alkaline Phosphatases From Various Sources

3.1 INTRODUCTION

Alkaline phosphatase is a ubiquitous enzyme, and certainly one of the most extensively studied[1]. Although all alkaline phosphatases perform the same function, the hydrolysis of a phosphoester bond, they differ in many respects, depending on their source. Structurally, they have different amino acid sequences, although they invariably share the amino acid sequence of the active site[2]. The molecular weights for the native enzymes vary quite significantly, from about 68 kDa for a *Pseudomonas aeruginosa* to possibly more than 200 kDa for an isoform of human placental alkaline phosphatase[3]. Some isozymes and isoforms in their active configurations are monomers, many are dimers, and some have been thought to work as tetramers. It appears that all alkaline phosphatases use metal ions (Zn^{2+} and Mg^{2+}) although the extent to which they are bound may vary[4]; phosphorylation at the active site also seems to be a common thread. Some alkaline phosphatases are heavily modified by the addition of carbohydrates (up to 30% of molecular weight) or lipids, while others have no modifications of this sort[2]. Alkaline phosphatases can exhibit very different specific activities, even in their various optimal working conditions. This is true for enzymes coming from different species, as well as different organs within a species, and even different regions within a given organ. As the name suggests, alkaline phosphatases prefer basic conditions for their activity, and though that is true for all sources of enzyme, the actual pH optimum for any alkaline phosphatase depends on factors such as substrate type and concentration, buffer structure and concentration, and temperature. The range for optimal pH values can be roughly described as 7.5 - 11.00, with mammalian isoenzymes preferring, in general, the higher values[5]. The stabilities of alkaline phosphatases are also varied; some isozymes are extremely hardy, resisting heat, acids and alkali, and many denaturing agents, while others do not tolerate even slight disturbances in the environment. With an enzyme this prevalent and so varied, there will always be room for more studies of alkaline phosphatase.

One of the more popular alkaline phosphatases in use today is calf intestinal alkaline phosphatase. Although performing much the same function as its *E. coli* counterpart, it is quite a different enzyme. The calf intestinal isozyme is one of four isoenzymes found in most mammals: intestinal, placental, placental-like, and tissue non-specific (bone/kidney/liver). It is the product of the ALPI gene. The enzyme is found in the brush border mucosa epithelial cells on the absorptive side of the intestine, pointing to its role in the transport of nutrients across the epithelial membrane[6]. It is attached to the membrane through a glycosylphosphatidylinositol (GPI) anchor[7]. Calf intestinal alkaline phosphatase is much more typical of the alkaline phosphatase family than the *E. coli* isozyme, with its pH optimum near 10 and its optimum temperature near 40°C. As with most alkaline phosphatases, the active enzyme is a dimer with a molecular weight estimated at between 140-160 kDa, including two carbohydrate chains[8-10]. The molecular weight of the polypeptide chain in its mature form is calculated to be 105.1 kDa. The carbohydrate content is estimated at 12% to 22% of the molecular weight of the enzyme[2]. This includes hexose, hexosamine, and possibly sialic acid. Although the enzyme has not yet been crystallized, it has been fully sequenced, and the partial sequence around the active site is known to be analogous to all alkaline phosphatases (Asp-Ser-Ala). The full sequence is shown in **Figure 3.1**. There is also a high degree of homology in the overall amino acid content between calf intestinal and other alkaline phosphatase isozymes[2]. The 27 amino acid long propeptide, or the signal sequence at the C-terminus, is cleaved off after synthesis of the polypeptide chain at the ribosomes of the rough endoplasmic reticulum (ER), coupled to the attachment of the GPI moiety[7]. The 19 amino acid long signal sequence at the N-terminus is cleaved off in the mature form of the enzyme, after it has been transported to its destination at the membrane. Just as the *E. coli* alkaline phosphatase, the calf intestinal isoform is phosphorylated at the active site and it possesses the same metals, although the number of ions of each (Mg^{2+} and Zn^{2+}) in

Figure 3.1 The amino acid sequence of a bovine intestinal alkaline phosphatase subunit precursor including the signal sequence, starting at the N-terminus. Adapted from Expasy's Swiss-Prot database at http://expasy.cbr.nrc.ca/.

M(1) - G(19) = signal sequence

L(20) - **D**(506) = main chain

A(507) - Y(533) = propeptide, removed in mature form

S(111) = active site, phosphorylation site

D(506) = glycosylphosphatidylinositol (GPI) anchor

N(268, 429) = glycosylation sites

```
          10          20          30          40          50
           |           |           |           |           |
MQGACVLLLL GLHLQLSLGL VPVEEEDPAF WNRQAAQALD VAKKLQPIQT
          60          70          80          90         100
           |           |           |           |           |
AAKNVILFLG DGMGVPTVTA TRILKGQMNG KLGPETPLAM DQFPYVALSK
         110         120         130         140         150
           |           |           |           |           |
TYNVDRQVPD SAGTATAYLC GVKGNYRTIG VSAAARYNQC KTTRGNEVTS
         160         170         180         190         200
           |           |           |           |           |
VMNRAKKAGK SVGVVTTTRV QHASPAGAYA HTVNRNWYSD ADLPADAQMN
         210         220         230         240         250
           |           |           |           |           |
GCQDIAAQLV NNMDIDVILG GGRKYMFPVG TPDPEYPDDA SVNGVRKRKQ
         260         270         280         290         300
           |           |           |           |           |
NLVQAWQAKH QGAQYVWNRT ALLQAADDSS VTHLMGLFEP ADMKYNVQQD
         310         320         330         340         350
           |           |           |           |           |
HTKDPTLQEM TEVALRVVSR NPRGFYLFVE GGRIDHGHHD DKAYMALTEA
         360         370         380         390         400
           |           |           |           |           |
GMFDNAIAKA NELTSELDTL ILVTADHSHV FSFGGYTLRG TSIFGLAPSK
         410         420         430         440         450
           |           |           |           |           |
ALDSKSYTSI LYGNGPGYAL GGGSRPDVND STSEDPSYQQ QAAVPQASET
         460         470         480         490         500
           |           |           |           |           |
HGGEDVAVFA RGPQAHLVHG VEEETFVAHI MAFAGCVEPY TDCNLPAPTT
         510         520         530
           |           |           |
ATSIPDAAHL AASPPPLALL AGAMLLLLAP TLY
```

the molecule is not certain. The enzyme is more than 100 times more active under optimal conditions than the *E. coli* isoform[11,12]. This makes it especially suitable for immunological applications such as enzyme linked immunosorbent assays (ELISAs). There have been attempts at purification of calf intestinal alkaline phosphatase isoforms[13-15]. Anywhere between 1 and 5 isoforms have been seen in DEAE-cellulose chromatography, starch-gel electrophoresis, and in IEF gels[9,13-16]. The difference between the isoforms was never determined but it is more than likely that it could be attributed to glycosylation patterns. There has been some evidence that specific activity of calf intestinal alkaline phosphatase increases with increasing hexose/hexosamine content[9]. Previous work by Craig *et al.* on single molecules of this enzyme showed a large degree of heterogeneity in the activities of individual molecules[17-19]. This was attributed to post-translational glycosylation.

In addition to *E. coli* and calf intestinal alkaline phosphatases, isozymes from 4 other sources were briefly looked at in the course of this research: bovine kidney, porcine kidney, human placenta, and shrimp alkaline phosphatases.

Bovine kidney alkaline phosphatase is a tissue non-specific bovine isozyme, the product of the ALPL gene. It is found in the brush border epithelial cells of renal proximal tubules; again, its role there is tied to the transport of nutrients across the epithelial membrane. Its sequence has been deduced and is shown in **Figure 3.2**. The polypeptide chain includes a 17 amino acid signal sequence, removed in the mature form. The enzyme is a dimer with a molecular weight of about 172 kDa; it contains 4.5 g-atoms Zn/mol protein; 4 Zn^{2+}/dimer are necessary for its activity[20]. The enzyme also contains Mg^{2+}; this cation has a stimulatory effect on bovine kidney alkaline phosphatase - up to 60 fold activation has been achieved with high Mg^{2+} concentrations[21]. This alkaline phosphatase is also a glycoprotein, with up to five potential N-linked carbohydrate chains. These are thought to include hexosamine, sialic acid, and neutral CHO[20]. As with the previous examples of the alkaline

Figure 3.2 The amino acid sequence of a bovine kidney alkaline phosphatase subunit precursor including the signal sequence, starting at the N-terminus. Adapted from Expasy's Swiss-Prot database at http://expasy.cbr.nrc.ca/.

M(1) - S(17) = signal sequence

L(18) - F(524) = main chain

S(110) = active site, phosphorylation site

N(140, 230, 271, 303, 430) = glycosylation sites

```
          10         20         30         40         50
           |          |          |          |          |
MISPFLLLAI GTCFASSLVP EKEKDPKYWR DQAQQTLKNA LRLQTLNTNV
          60         70         80         90         100
           |          |          |          |          |
AKNVIMFLGD GMGVSTVTAA RILKGQLHHS PGEETKLEMD KFPYVALSKT
         110        120        130        140        150
           |          |          |          |          |
YNTNAQVPDS AGTATAYLCG VKANEGTVGV SAATQRSQCN TTQGNEVTSI
         160        170        180        190        200
           |          |          |          |          |
LRWAKDAGKS VGIVTTTRVN HATPSASYAH SADRDWYSDN EMPPEALSQG
         210        220        230        240        250
           |          |          |          |          |
CKDIAYQLMY NIKDIEVIMG GGRKYMFPKN RTDVEYELDE KARGTRLDGL
         260        270        280        290        300
           |          |          |          |          |
NLIDIWKSFK PKHKHSHYVW NRTDLLALDP HSVDYLLGLF EPGDMQYELN
         310        320        330        340        350
           |          |          |          |          |
RNNATDPSLS EMVEMAIRIL NKNPKGFFLL VEGGRIDHGH HEGKAKQALH
         360        370        380        390        400
           |          |          |          |          |
EAVEMDQAIG QAGAMTSVED TLTVVTADHS HVFTFGGYTP RGNSIFGLAP
         410        420        430        440        450
           |          |          |          |          |
MVSDTDKKPF TAILYGNGPG YKVVGGEREN VSMVDYAHNN YQAQSAVPLR
         460        470        480        490        500
           |          |          |          |          |
HETHGGEDVA VFAKGPMAHL LHGVQEQNYI PHVMAYAACI GANRDHCASA
         510        520
           |          |
SSSGSPSPGP LLLLLALLPL GSLF
```

phosphatase family, the bovine kidney isozyme is phosphorylated at its active site, serine; the sequence around the active site is homologous to that of other alkaline phosphatases[2]. The enzyme is thought to exist in at least 2 isoforms, distinguishable through IEF. The difference between the isoforms is thought to be due to sialic acid content - upon neuraminidase treatment, the electrophoretic differences between the isoforms disappear[20]. The activity of bovine kidney alkaline phosphatase is one of the highest in mammalian tissues; it is less than that of calf intestinal isozyme, but more than that of porcine kidney alkaline phosphatase[22,23]. The optimum pH for this enzyme is around 10[24] and optimum temperature around 40°C[21]. The enzyme is relatively resistant to tryptic digestion[23].

The next alkaline phosphatase used in our laboratory is the porcine kidney alkaline phosphatase. This is an enzyme analogous to the bovine kidney counterpart. Its amino acid sequence is as of yet unknown. The molecular weight of an active dimer has been estimated at 156-185 kDa[25-27], including approximately 30% carbohydrate content, mainly sialic and acetyl neuraminic acids. The enzyme needs 4 Zn^{2+} and 2 Mg^{2+} ions for activity[26]. The optimum pH and temperature for this enzyme are thought to be 9.8-10.0 and approximately 37°C, respectively[28]. It is relatively heat labile, as compared to the *E. coli* and calf intestinal alkaline phosphatases. This is an example of a very active alkaline phosphatase[22]; it is also resistant to trypsin. Several isoforms have been found for this enzyme differing in their heat stability, resistance to urea and pH. As with the bovine kidney counterpart, the electrophoretic differences between the isoforms disappear upon treatment with neuraminidase; the isoforms become identical in all respects under investigation[26].

The human placental alkaline phosphatase is the only human source alkaline phosphatase isozyme which I will discuss. It is the product of the ALPP (also known as PLAP) gene. There are at least 3 different types of the placental alkaline phosphatase (types 1 - 3, also known as (S)low-, (I)ntermediate-, and (F)ast-moving)[29], differing

from each other by substitutions of amino acids at seven sites[30-32]. Furthermore, only seven amino acids distinguish the placental and placental-like (also known as germ cell) isozymes, which are known to be coded for by different genes[33]. The enzyme has been sequenced (see **Figure 3.3** for a complete amino acid sequence of type 1 placental alkaline phosphatase) and very recently it has become the first eukaryotic alkaline phosphatase to have its crystal structure solved[34]. The enzyme is a dimer of two identical subunits, each containing 2 Zn^{2+}, 2 Mg^{2+}, and one phosphate ion at the active site. The molecular weight of the enzyme, by various estimates, is thought to be 116-125 kDa[35-37]. The placental enzyme has two glycosylation sites, and the carbohydrate content, estimated at 25-30% of the molecular weight of the enzyme, is thought to include glucosamine, galactosamine, mannose, fucose, and sialic acid[38]. It is also attached to cell membrane through a GPI anchor, added to the C-terminus following the removal of a 29 amino acid long signal sequence[39]. A 22 amino acid long signal sequence also exists at the N-terminus and is removed once the enzyme reaches its destination at the membrane inner surface. The enzyme has temperature and pH optima of around 40°C and 10.3 - 10.8[40], respectively. It is also thought to be relatively stable with respect to pH, temperature, and dissociation by chemical means[38]. Eight separate forms of human placental alkaline phosphatase have been obtained by IEF in a sucrose density gradient by Khattab and Pfleiderer[15]; this number was reduced to 4 upon neuraminidase treatment. The placental enzyme has also been found to be particularly resistant to denaturation during electrophoresis.

Shrimp alkaline phosphatase is the last of the alkaline phosphatases assayed on a single molecule basis in my research. Relatively little is known about this enzyme. A group in China has studied alkaline phosphatases from two different shrimp species: *Penaeus japonicus* and *Penaeus monod*[41,42]. They found the alkaline phosphatases from these two species to be different in a few aspects. The molecular weight was estimated only for *P. japonicus*, and it was found to be 110 kDa. Native PAGE

Figure 3.3 The amino acid sequence of a human placental type 1 alkaline phosphatase subunit precursos including the signal sequence, starting at the N-terminus. Adapted from Expasy's Swiss-Prot database at http://expasy.cbr.nrc.ca/.

M(1) - G(22) = signal sequence

I(20) - D(506) = main chain

A(507) - P(535) = propeptide, removed in mature form

S(114) = active site, phosphorylation site

D(506) = glycosilphosphatidylinositol (GPI) anchor

N(144, 271) = glycosylation sites

```
          10          20          30          40          50
           |           |           |           |           |
MLGPCMLLLL LLLGLRLQLS LGIIPVEEEN PDFWNREAAE ALGAAKKLQP
          60          70          80          90         100
           |           |           |           |           |
AQTAAKNLII FLGDGMGVST VTAARILKGQ KKDKLGPEIP LAMDRFPYVA
         110         120         130         140         150
           |           |           |           |           |
LSKTYNVDKH VPDSGATATA YLCGVKGNFQ TIGLSAAARF NQCNTTRGNE
         160         170         180         190         200
           |           |           |           |           |
VISVMNRAKK AGKSVGVVTT TRVQHASPAG TYAHTVNRNW YSDADVPASA
         210         220         230         240         250
           |           |           |           |           |
RQEGCQDIAT QLISNMDIDV ILGGGRKYMF RMGTPDPEYP DDYSQGGTRL
         260         270         280         290         300
           |           |           |           |           |
DGKNLVQEWL AKRQGARYVW NRTELMQASL DPSVTHLMGL FEPGDMKYEI
         310         320         330         340         350
           |           |           |           |           |
HRDSTLDPSL MEMTEAALRL LSRNPRGFFL FVEGGRIDHG HHESRAYRAL
         360         370         380         390         400
           |           |           |           |           |
TETIMFDDAI ERAGQLTSEE DTLSLVTADH SHVFSFGGYP LRGSSIFGLA
         410         420         430         440         450
           |           |           |           |           |
PGKARDRKAY TVLLYGNGPG YVLKDGARPD VTESESGSPE YRQQSAVPLD
         460         470         480         490         500
           |           |           |           |           |
EETHAGEDVA VFARGPQAHL VHGVQEQTFI AHVMAFAACL EPYTACDLAP
         510         520         530   535
           |           |           |     |
PAGTTDAAHP GRSVVPALLP LLAGTLLLLE TATAP
```

showed only a single band for this enzyme suggesting homogeneity with respect to size. The enzyme is a dimer. The weight of deglycosylated subunit was 33 kDa, suggesting a carbohydrate content of approximately 33%. The carbohydrates are thought to be N-linked sialic acid. The isoelectric points were found to be 7.6 and 6.9 for the *P. japonicus* and *P. monod*, respectively. There is no information as to the pI heterogeneity of either isozymes. *P. japonicus* alkaline phosphatase is attached to the cell membrane through a glycophosphatidylinositol anchor; most likely the same is true for the other species. This enzyme was also found to be heat stable while that of *P. monod* was heat-labile. The pH optima for the two isozymes also differed: 10.0 for *P. japonicus* and 9.0 for *P. monod*.

Some of the characteristics of the alkaline phosphatase isoenzymes discussed above are gathered in **Table 3.1**.

In addition to exploring the alkaline phosphatase family in the context of single molecule enzymology, some work was done in an attempt to improve assay conditions for working with mammalian alkaline phosphatases. Since borate is a known inhibitor of calf intestinal alkaline phosphatase[43], two different buffers were tested for their compatibility with CE. These are ethylaminoethanol (EAE) and 2-amino-2-methyl-1-propanol, both amino alcohols which at relatively high concentrations stimulate alkaline phosphatase activity by serving as phosphoacceptors[44]. In addition, their pK_a values are closely matched to optimum pH values of mammalian enzymes. Buffer additives were also looked at in this light. The results of these preliminary experiments charted the course for most of the work presented in this thesis.

3.2 PRELIMINARY STUDIES ON THE CALF INTESTINAL ALKALINE PHOSPHATASE SYSTEM

The calf intestinal alkaline phosphatase was the first enzyme used in our laboratory to develop on-capillary single enzyme molecule assays. The progress and

Table 3.1 Comparison of some properties of alkaline phosphatases from various sources. Human placenta AP describes all three types of the placental enzyme. Shrimp AP describes both shrimp species: *Penaeus japonicus* and *P. monod.*
(NA = not available)

	Source of Alkaline Phosphatase					
Property	*E. coli*	Calf Intestine	Bovine Kidney	Porcine Kidney	Human Placenta	Shrimp
gene	*pho*A	ALPI	ALPL	NA	ALPP	NA
# amino acids	449/450	487	507	NA	484	NA
MW (kDa)	89	140-160	172	156-185	116-140	110
CHO content	none	12 - 22%	15%	30%	25-30%	33%
GPI anchor	no	yes	no	no	yes	yes
pI	5.1 -5.5	4.2-5.8			4.8	6.9 -7.6
no. of isoforms	3	≤ 5	min. 2	several ?	≤ 8	NA
optimum T	≤ 80°C	≈ 40°C	≈ 40°C	≤ 40°C	≈ 40°C	NA
optimum pH	≈ 8	10.5		9.8 - 10	10.3	9.0-10.0

results of that work can be found in a number of publications by Craig *et al.* These studies showed a relatively large heterogeneity in the catalytic behaviour of individual enzyme molecules; this heterogeneity was attributed to post-translational glycosylation of this enzyme. My research sought to better define the relationship between isozyme structure and its catalytic activity. Initially the use of calf intestinal alkaline phosphatase was to be continued, with some changes to the assay procedure and some further purification of the enzyme preparation, which would hopefully result in a more structurally homogeneous enzyme. The changes proposed for the assay included switching to a different buffer, adding the two essential metal cations to the buffer, and switching to a smaller diameter capillary.

The first change was dictated by the fact that borate buffer is a known weak competitive inhibitor of this particular alkaline phosphatase. Since our single molecule experiments can only show the static disorder in the behaviour of enzymes, it was unlikely that competitive inhibition by borate would manifest itself through heterogeneous, time-averaged behaviour of individual enzyme molecules. Nevertheless, a move from an inhibitory to a stimulatory buffer had at least one obvious advantage - a significant shortening of assay times. Adding metal cations to the reaction buffer, the second proposed change, could also help in the enzymatic assays. The use of these cations in assay mixtures is recommended by routine users of this enzyme. Although the affinity of alkaline phosphatase for Mg^{2+} and Zn^{2+} is relatively high, and so it was unlikely that the metals would be lost during the assay, providing the metals in the reaction medium could possibly stimulate the enzyme further. Once again, this would allow for shortening of the reaction time.

It was also important to purify the enzyme to structural homogeneity. This was hoped to be achieved in two ways. One, the enzyme should be purified into its isoforms, which could then be studied separately to find out if the catalytic heterogeneity persisted within each isoform. Two, the enzyme should be

deglycosylated to find out if oligosaccharides were in fact responsible for this heterogeneity.

Moving to a smaller i.d. capillary was designed to help achieve better sensitivity without changing the experimental set-up. Successful implementation of these changes would allow for faster and more accurate determination of the catalytic behaviour of alkaline phosphatase.

3.2.1 Instruments

All CE work was done on the in-house built capillary electrophoresis instrument with laser induced fluorescence detection (CE-LIF), utilizing a sheathflow cuvette and a Peltier-driven heater/cooler unit. The instrument has been described previously. In the case of preliminary work on buffer compatibility and in early calf intestinal AP assays the heater was not yet used. The fused silica capillaries from Polymicro Technologies (Phoenix, AZ) were 10 μm i.d., approx. 150 μm o.d., of variable length. For the study of smaller diameter capillaries, 2 and 5 μm i.d., 145 μm o.d. fused silica capillaries, also from Polymicro Technologies, were used. In all capillaries the polyimide was removed from the detection end to about 1-3 mm with a gentle flame.

IEF of calf intestinal alkaline phosphatase was done on the Pharmacia PhastSystem (Pharmacia LKB, Uppsala, Sweden), a horizontal bed apparatus, as described in **Section 2.2.1**. Molecular weight determination following deglycosylation was done using a BioRad Mini Protean II vertical gel electrophoresis cell (Bio-Rad Laboratories, Hercules, CA), as described in **Section 2.2.2**.

3.2.2 Materials

Bovine calf intestinal alkaline phosphatase (CI-AP, cat. # P 7923) was purchased from Sigma (Sigma-Aldrich Canada Ltd., Oakville, ON) as a solution in

50% glycerol containing 5 mM $MgCl_2$ and 0.1 mM $ZnCl_2$, pH 7.5. The lot (#17H0204) was described as having 21 mg enzyme/mL at 4,790 units/mg. The unit definition for this enzyme is given as: one unit is the amount of enzyme that will hydrolyze 1 µmol of p-nitrophenyl phosphate (pNPP) per minute at 37°C in 1 M diethanolamine (DEA) buffer, pH 9.8, containing 0.5 mM $MgCl_2$, and 15 mM pNPP.

2-Amino-2-methyl-1-propanol (2A2M1P) and ethylaminoethanol (EAE) were purchased from Sigma as SigmaUltra reagents. $MgCl_2$ and $ZnCl_2$ were purchased from Aldrich Chemical Co. (Milwaukee, WI) as certified, anhydrous, 99.99% and 99.999% pure, respectively. NaOH, HCl, and boric acid were of analytical grade. Glycerol was purchased from Sigma. Enzymatic Deglycosylation Kit (for enzymatic removal of Asn and Thr/Ser linked oligosaccharides) was purchased from Glyko, Inc. (Novato, CA). The kit contains O-Glycosidase DS, NANase II, PNGase F, as well as required buffers, reagents, and a bovine fetuin control.

IEF of alkaline phosphatase was performed on the Pharmacia PhastSystem. The PhastGels (pH range 4-6.5 and 5-8) were purchased from Pharmacia (Amersham Pharmacia Biotech, Uppsala, Sweden).

3.2.3 Experimental Methods

Experimental conditions will be described separately for buffer compatibility and instrumental modifications (buffer type and metal content, capillary size study), deglycosylation, and isoelectric focusing.

3.2.3.1 Buffer Compatibility and Instrument Modifications

In view of the inhibitory effect of borate buffer on calf intestinal AP, attempts were made at adapting two buffers recommended for use with alkaline phosphatase assays. Ethylaminoethanol (EAE) and 2-amino-2-methyl-1-propanol (2A2M1P) at various concentrations were used to generate Ohm's plots, in order to find out the best

combination of the highest acceptable concentration of buffer and the highest electric field that could be used in CE assays on single enzyme molecules. EAE at concentrations of 100 mM, 200 mM and 950 mM, and 2A2M1P at concentrations of 100 mM and 1.0 M were run at electric fields starting with 10 V/cm, then 50 V/cm, and then increasing stepwise (50 V/cm step) to 450 V/cm. The current was allowed to stabilize before taking a reading. Sheath flow buffer matched the running buffer. All buffers had their pH adjusted to 10.0 and were filtered twice through 0.22 μm Millex membranes (Millipore Corp., Bedford, MA) before use.

For the study of buffer additives, highly pure $MgCl_2$ and $ZnCl_2$ at a concentration of 1 mM were added to borate buffer (as used in previous assays), injected frontally onto the capillary, and allowed to run until current became stable.

In the study of capillary size, 2 and 5 μm i.d. capillaries were installed on the instrument and filled with borate buffer. The buffer was electrophoresed at a constant voltage. Stability of current was observed as indicative of capillary's performance.

3.2.3.2 Enzymatic Deglycosylation of Calf Intestinal Alkaline Phosphatase

The enzymatic deglycosylation of calf intestinal alkaline phosphatase (CI-AP) was achieved using a deglycosylation kit. According to the Glyko product specifications sheet, the kit will remove all Asn-linked oligosaccharides from glycoproteins using the enzyme PNGase F while the combination of enzymes NANase II and O-Glycosidase DS will remove all Ser/Thr linked Gal(β1,3)GalNAc(α1) and all sialic acid substituted Gal(β1,3)GalNAc(α1). Other solutions included with the kit are:

- 5× Reaction Buffer B = 250 mM Na_3PO_4, pH 6.0
- pH Adjustment Buffer = 0.5 M Na_2HPO_4
- SDS/β-mercaptoethanol

Full and partial (using only NANase II to remove Asn linked oligosaccharides) deglycosylations were performed. CI-AP is thought to possess only N-linked carbohydrates, which would mean that a partial deglycosylation should be sufficient. Full deglycosylation was performed under both denaturing and non-denaturing conditions. The full deglycosylation protocol was as follows:

1. Mix 6 µL CI-AP, 12 µL dd-H_2O, and 6 µL Reaction Buffer B,
2. Mix in 3 µL NANase II - incubate at 37°C for 60 min.,
3. Mix in 3 µL O-Glycosidase DS - incubate at 37°C for 180 min.,
4. Mix in 15 µL dd-H_2O and 15 µL pH Adjustment Buffer,
5. Mix 20 µL of above reaction mixture with 1.5 µL SDS/b-mercaptoethanol, heat at 100°C fir 5 min., then cool on ice; mix in 1.5 µL PNGase F and incubate at 37°C for 180 min. - THIS IS THE DENATURED SAMPLE
6. To the remaining 40 µL of the reaction mixture from steps 1-4 add 2.0 µL PNGase F, incubate at 37°C for 24 hrs - THIS IS THE NON-DENATURED SAMPLE

The partial deglycosylation protocol was as follows:

1. Mix 5 µL CI-AP, 9 µL dd-H_2O, and 4 µL Reaction Buffer B,
2. Mix in 2 µL NANase II,
3. Incubate at 37°C for 60 min.

In each case, a blank was prepared by mixing in an equivalent amount of dd-H_2O instead of CI-AP. The success of the procedure was judged by running the deglycosylated enzyme, the blanks, and a control (a 1/10 dilution of CI-AP in running buffer) by SDS-PAGE.

3.2.3.3 Isoelectric Focusing of Calf Intestinal Alkaline Phosphatase

For the IEF of CI-AP, a small amount of the enzyme was applied to a Pharmacia PhastGel and run on the PhastSystem electrophoresis instrument and the Separation

Technique File No. 100. The enzyme was diluted 100-fold. The gels were developed using silver staining according to a modification of the Pharmacia Development Technique File No. 210. The separation and development are also described in **Section 2.2.1**.

3.2.4 Results of Preliminary Work

The results of the preliminary work with calf intestinal alkaline phosphatase are presented here grouped in three sections, analogous to those in the Experimental Methods section.

3.2.4.1 Results of Buffer Compatibility Studies and Instrumental Modifications

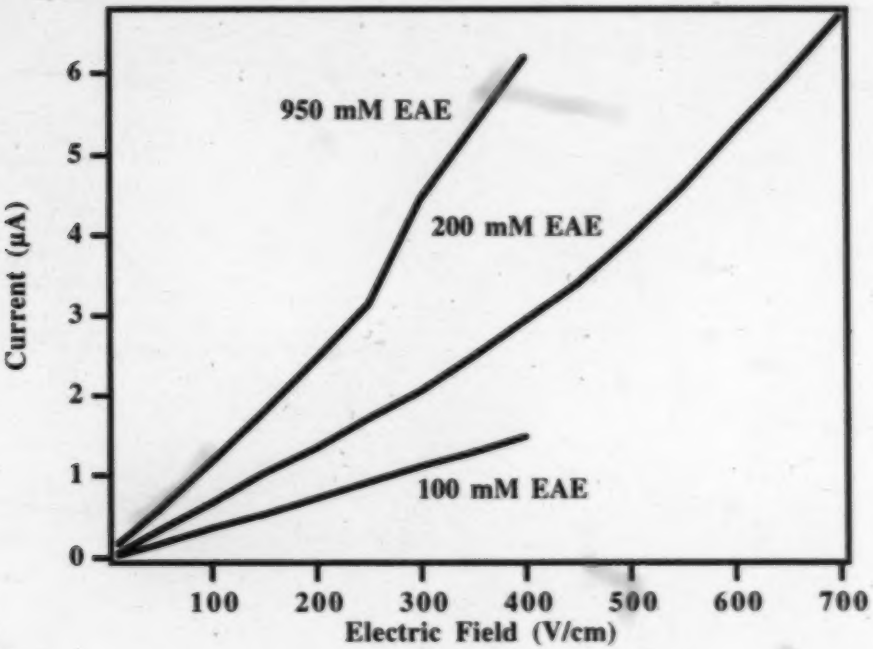
The result of the study on the compatibility of EAE and 2A2M1P with the CE set-up in use are shown in **Table 3.2** and **Figures 3.4 - 3.5**. Although it appeared that the two aminoalcohols were compatible with CE, as judged by the stabilization of current during the stepwise increase of the electric field, a number of problems were observed. The background was relatively high with these two buffers as compared to that observed when using borate (approximately 8-10 times higher for EAE and 2-3 times higher for 2A2M1P). Capillaries seemed to become plugged regularly despite double filtration of buffers using 0.22 μm membranes. When using either of the buffers in conjunction with fluorescein, a fluorescent substance used for daily instrument alignment, getting rid of the residual fluorescein was impossible by electrophoretic means; syringe flush had to be used. Finally, the capillaries used with these buffers had an unusually short life spans.

The use of Mg^{2+} and Zn^{2+} in bare fused silica capillaries proved to be detrimental to their stability. Addition of these metals into the running buffer resulted in

Table 3.2 Results of the buffer compatibility study on EAE and 2A2M1P. 200 mM 2A2M1P also contains 10% DMSO. "Equilibr." means that current was not stable. Lack of value (–) means that a reading was not taken.

Electric Field	Current (μA)					
	ethylamino ethanol			2-amino-2-methyl-1-propanol		
(V/cm)	100 mM	200 mM	950 mM	100 mM	200 mM	1.0 M
10	0.02	0.05	0.15	0.01	0.01	0.18
20	–	–	–	–	0.05	0.38
30	–	–	–	–	0.08	0.57
50	0.16	0.33	0.59	0.13	0.15	–
100	0.35	0.68	1.19	0.27	0.35	1.81
150	0.54	1.03	1.82	0.52	0.54	2.72
200	0.73	1.36	2.46	0.75	0.74	equilibr.
250	0.92	1.72	3.14	1.00	0.94	equilibr.
300	1.12	2.07	4.45	1.29	1.15	equilibr.
350	1.31	2.49	5.33	1.58	1.35	–
400	1.51	2.94	6.20	1.90	1.56	–
450	–	3.41	–	2.26	–	–
500	–	3.96	–	–	–	–
550	–	4.58	–	–	–	–
600	–	5.29	–	–	–	–
650	–	5.98	–	–	–	–
700	–	6.72	–	–	–	–

Figure 3.4 Ohm's plots for ethylamino ethanol (EAE), pH 10.0, and 2-amino-2-methyl-1-propanol (2A2M1P), pH 10.0.



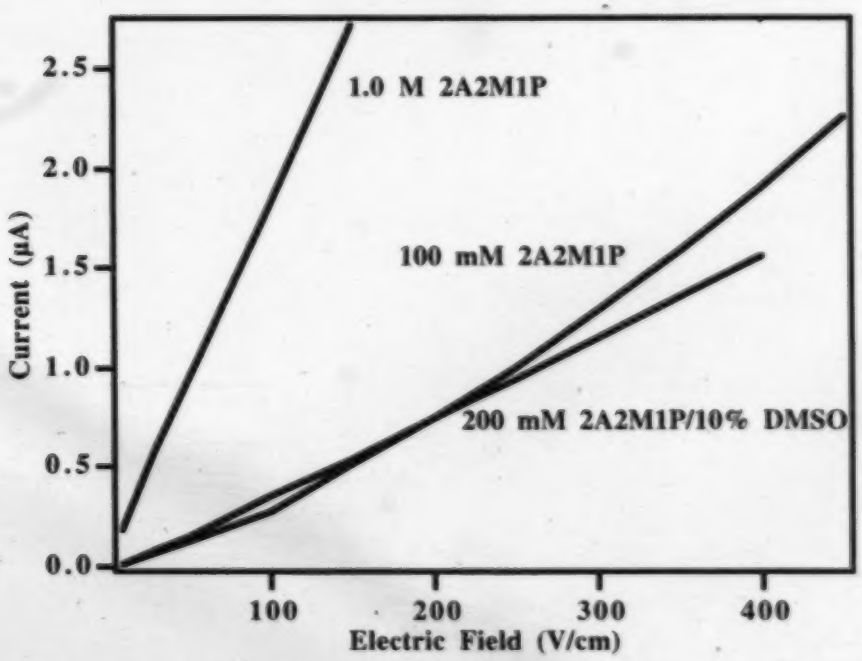
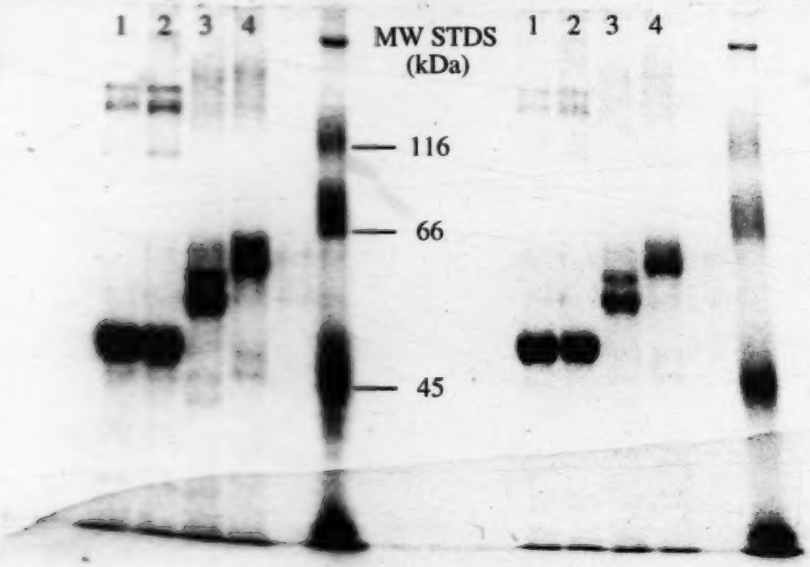
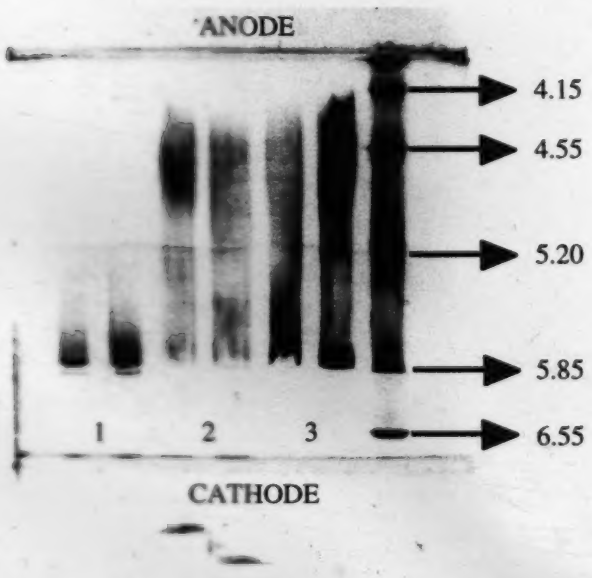


Figure 3.5 SDS-PAGE (**a**) and IEF (**b**) of CI-AP and deglycosylated CI-AP.
a) sample 1&2 - EC-AP, sample 3 - deglycosylated CI-AP, sample 4 - native CI-AP;
b) sample 1- native CI-AP, sample 2- deglycosylated CI-AP (no denaturation), sample 3 - denatured, deglycosylated CI-AP

a)



b)



current drift, which continued for as long as the metal containing buffer was electrophoresed through the capillary. Attempts at pre-incubation of the capillary with a relatively high concentration of Mg^{2+} and Zn^{2+} resulted in extremely high current which could not be sustained by the capillary.

The use of small diameter capillaries was an utter failure. The capillaries were unable to carry any current, presumably because of plugging. Again, double filtration of all buffers did not prevent this problem from occurring.

3.2.4.2 Deglycosylation of Calf Intestinal Alkaline Phosphatase

Only the full deglycosylation under denaturing conditions was successful at removing the carbohydrate moieties off the polypeptide chain as evidenced by the reduction in the molecular weight of the CI-AP subunit (see **Figure 3.5 (a)**). Full deglycosylation under non-denaturing conditions and partial deglycosylation (also done without denaturation) resulted in subunits with the same MW as the native enzyme subunit (results are not shown). **Figure 3.5 (b)** shows the results of isoelectric focusing of native and deglycosylated alkaline phosphatase. There is very little difference between the CI-AP deglycosylated under non-denaturing conditions and native alkaline phosphatase. In contrast, the IEF of denatured, deglycosylated CI-AP suggests a reduction in the number of isoforms as evidenced by a shorter smear in the gel.

3.2.4.3 Isoelectric Focusing of Calf Intestinal Alkaline Phosphatase

Despite some previous successes with IEF of CI-AP, all attempts at obtaining well focused bands of this enzyme on polyacrylamide IEF gels failed. Invariably the enzyme was found spread out along the gel lane - a broad smear as opposed to tight bands. A shorter smear was found to be produced by the denatured, deglycosylated CI-AP. A picture of a silver stained IEF gel with CI-AP is shown in **Figure 3.5 (b)**.

3.2.5 Discussion

Attempts at improving assay conditions through a combination of buffer type and additive changes and instrumental design changes were unsuccessful. Although the amino alcohols could be used alone in the capillary, provided that their concentration was kept at 100-200 mM, there were other problems associated with their use. The high backgrounds obtained with EAE were probably due to this solution's yellowish hue (likely due to impurities). The plugging of the capillaries could have been due to small impurities in the buffers which might have aggregated under the influence of an electric field. It could have also been caused by aggregation of the aminoalcohol molecules themselves. In an aqueous solution both EAE and 2A2M1P exist in equilibrium with their protonated forms, which may adsorb onto the negatively charged capillary walls, and further aggregate through hydrogen bonding. To find out the proportion of the protonated form in either case, we can use a rearranged form of the equilibrium expression for the dissociation of a protonated aminoalcohol:

$$\frac{K_a}{[H^+]} = \frac{[B]}{[BH^+]} \qquad \textbf{(Equation 3.1)}$$

where B is the aminoalcohol, BH^+ is its protonated form, K_a is the dissociation constant of the protonated aminoalcohol, and $[H^+]$ is the concentration of the hydronium ion. $[B]/[BH^+]$ is the ratio of deprotonated to protonated forms of the amino alcohol. For EAE ($pK_a = 9.9$) and 2A2M1P ($pK_a = 9.3$) at pH = 10.0, these ratios are 1.3 and 5.0, respectively. This suggests the amount of the protonated form to be around 44% for EAE, and around 17 % for 2A2M1P. These are appreciable amounts, likely to cause problems at the capillary wall when high concentrations of aminoalcohols are used.

Despite the difficulties with current at high aminoalcohol concentrations in the running buffer, the use of 2A2M1P at intermediate concentration of 200 mM was explored a little further. The buffer was prepared at 200 mM, pH 10.0 with 1 mM AttoPhos™ (alkaline phosphatase substrate) and injected onto a capillary at 400 V/cm

for 300 s. After a 20 min. incubation, the contents of the capillary were swept past the detector at 400 V/cm. Unfortunately, this buffer also proved to be difficult for single enzyme molecule work. There was a great deal of enzymatic contamination, which could not be fully removed in any way (autoclaving, regular flushes of the capillary with NaOH). The product and substrate plateaus could not be sufficiently resolved. The current was fluctuating appreciably, resulting in irreproducible runs. This required the use of frequent NaOH flushes of the capillary interior in order to remove adsorbed species, and so the run times were increased. Finally, adding DMSO (10%) to improve resolution between the plateaus resulted in lengthening the run times to a point when the runs were far too long to be practical.

Including the divalent cations of Zn and Mg in the running buffer did not improve the assay conditions either. A slow but steady current drift (upwards) was observed with even very small amounts of these metal cations included. This drift would eventually lead to a current breakdown. Pre-conditioning of the capillary with an overnight syringe flush of buffer containing large (100 mM) amounts of these cations, in an attempt to bind all the negative sites on the capillary wall, did not help. Current in the conditioned capillary was too high to be sustained. The addition of divalent cations to the running buffer had to be abandoned.

Finally, the use of very small i.d. capillaries proved to be impossible. Although their size would have contributed to sensitivity improvements in the measurements of single enzyme molecules' activities, it also made the experiments impractical. The capillaries became easily plugged, despite the best efforts to clean up buffers and samples before use.

Deglycosylation of calf intestinal alkaline phosphatase by enzymatic means was only partly successful. Full deglycosylation of denatured enzyme resulted in a decrease in the molecular weight of the subunit of no more than 10 kDa as compared to the native enzyme. As seen in the gel (**Figure 3.5 (a)**) however, not all of the enzyme

was fully deglycosylated. There are at least 2 bands visible in lane 3 and there can certainly be seen some staining in the region in that lane corresponding to native enzyme, as seen in lane 4. The full and partial deglycosylations of this enzyme under non-denaturing conditions showed no change in the MW (results not shown), nor in the IEF pattern of the enzyme (see **Figure 3.5 (b)**). In practical terms, enzymatic deglycosylation as performed here is not a useful tool in studying this enzyme by single molecule enzymology. Structural homogeneity was not achieved at all in species that remained enzymatically active, and was not even fully achieved with denatured species which were, in any case, useless in activity assays.

Similarly to deglycosylation, the isoelectric focusing of CI-AP was shown to be of no help in further studies of this enzyme's structure-activity relationship. The enzyme could not be focused on the polyacrylamide IEF gels into homogeneous, tight bands which could be later used to extract the isoforms for future studies by CE.

Overall, the calf intestinal alkaline phosphatase proved to be too complex of a molecule to achieve my goal. The inability to obtain structurally homogeneous isoforms meant that a different model should be found among other alkaline phosphatases.

3.3 A LOOK AT ALKALINE PHOSPHATASES FROM OTHER SOURCES

After the unsuccessful attempts at using calf intestinal alkaline phosphatase in the search for convincing evidence for the relationship between the enzyme's structure and its catalytic activity, my attention turned to the *E. coli* variant of the same enzyme (see **Chapter 2**). The success of that approach prompted a search for another mammalian alkaline phosphatase which could be used to look into the relationship between glycosylation and enzymatic activity. A successful candidate would have to be active, amply described in literature, and commercially available. In addition to calf

intestinal and *E. coli* alkaline phosphatases, four other potential candidates were looked at: bovine kidney AP (BK-AP), porcine kidney AP (PK-AP), human placenta AP (HP-AP), and shrimp AP (S-AP).

3.3.1 Materials and Instrumentation

All alkaline phosphatases described in this chapter were purchased from Sigma (Sigma-Aldrich Canada Ltd., Oakville, ON). The unit definition used to describe the activities of these preparations is as follows: one unit is the amount of enzyme that will hydrolyze 1 μmol of p-nitrophenyl phosphate (pNPP) per minute at 37°C in 1 M diethanolamine (DEA) buffer, pH 9.8, containing 0.5 mM $MgCl_2$, and 15 mM pNPP.

Bovine kidney alkaline phosphatase (BK-AP, cat. # P4653) was purchased as a lyophilized powder containing approximately 35% protein balanced with primarily Tris-citrate buffer salts. The lot was described as having 11 units/mg solid or 27 units/mg protein. This suggested a protein content of approximately 41%. The units used in this case were obtained in glycine buffer, pH 10.4. Glycine units correspond to approximately 1/5th of the DEA unit values.

Porcine kidney alkaline phosphatase (PK-AP, cat. # P4439) was purchased as a lyophilized powder containing approximately 90% protein balanced with Tris-HCl and NaCl. The lot (#72H01862) was described as having 142 units/mg solid or 155 units/mg protein. This corresponds to an approximate protein content of 92%.

Human placental alkaline phosphatase (HP-AP, cat. # P3895) was purchased as a lyophilized powder. The lot (#126H38614) was described as having 14 units/mg solid. The activity was assayed in glycine buffer, pH 10.4, corresponding to approximately 1/2 of that in DEA buffer.

Shrimp alkaline phosphatase (S-AP, cat. # P9088) was purchased as a solution in 50% glycerol, containing 25 mM Tris-HCl, pH 7.6, 1 mM $MgCl_2$, and 0.1 mM $ZnCl_2$. The lot (#69H0924) was described as having the protein content of 0.34

mg/mL and the activity in DEA buffer of 1,660 units/mL, or approx. 4,880 units/mg enzyme.

P-nitrophenol phosphate (pNPP) was obtained from Sigma Chemical Co.(St. Louis, MO) in tablet form at 15 mg pNPP/tablet. All buffers were prepared from analytical grade chemicals.

3.3.2 METHODS

The above alkaline phosphatases were assayed in bulk by a classical method using spectroscopic determination of free p-nitrophenol liberated from p-nitrophenol phosphate (p-NPP), and by single molecules using CE and the usual substrate employed, AttoPhos™™. Some of the enzymes were also subjected to SDS-PAGE and IEF.

3.3.2.1 Classical bulk assay for the determination of alkaline phosphatase activity

A standard spectrometric assay was performed on bulk solutions of all six alkaline phosphatase samples according to the method of Torriani[45]. A Hewlett-Packard 8451A spectrophotometer was used with a 1 mL quartz cuvette (1 cm path length) and set to 410 nm. Five different buffers were used in the assays:

1) 0.6 M Tris, pH 8.20 - the buffer recommended by Torriani in her method;

2) 1.0 M Tris, pH 8.00 - the recommended buffer for *E. coli* AP assays;

3) 100 mM Borate, pH 9.50 - CE assay running buffer, without organic modifier;

4) 85 mM borate/15% DMSO, pH 9.50 - CE assay running buffer;

5) 1.0 M 2A2M1P, pH 10.0 - the recommended buffer for mammalian AP assays.

The final concentration of substrate, p-nitrophenol phosphate (p-NPP), was 6.0 mM in all cases. All assays were done at 37°C over 5 minutes, with readings taken every 10 s. Each sample was measured in triplicate following a blank measurement where no

enzyme was added. The optimal sample dilution for the assays was determined experimentally.

3.3.2.2 Single molecule on-capillary assays of various source alkaline phosphatases

All alkaline phosphatases were also assayed on a single molecule basis on capillary. The instrument employed was that described in **Section 2.2.3** and the assays were performed according to the general method described in **Section 2.4.1**. All assays were done at 40°C.

3.3.2.3 SDS-PAGE and IEF of alkaline phosphatases from various sources

Bovine kidney AP and human placenta AP were subjected to SDS-PAGE on Bio-Rad ReadyGel, 12% Tris-HCl (Bio-Rad Laboratories, Hercules, CA). All four isozymes were subjected to IEF on Bio-Rad Ready Gel IEF, pH 5-8. Both were done using the Bio-Rad Mini Protean II apparatus, as described in **Section 2.2.2**. The SDS-PA gel was stained using Bio-Rad Silver Stain Plus. The IEF gel was stained using the AttoPhos™ activity based stain as described in **Section 2.3.1.1**. The buffer used in this case was 1.0 M 2A2M1P, pH 10.0 to support higher activities of the mammalian enzymes, which have pH optima around 10, and thus reduce the staining time and associated band diffusion.

3.3.3 Results

The results of the bulk assay of six different alkaline phosphatases (four described in this section plus calf intestinal and *E. coli* isozymes) in five different buffers are given in **Table 3.3**. The enzymatic activities are expressed in Torriani's units where one unit is defined as the amount of enzyme needed to bring about a change

Table 3.3 Results of bulk assays of alkaline phosphatases from various sources. The buffers were: 0.6 M Tris, pH 8.20; 1.0 M Tris, pH 8.00; 100 mM borate, pH 9.50; 85 mM borate/15% DMSO, pH 9.50; 1.0 M 2A2M1P, pH 10.0. One EU is the amount of enzyme needed to bring about a change in optical density of one unit per minute at 37°C, pH 8.2. EC = *E.coli*; HP = human placenta; BK= bovine kidney; PK = porcine kidney; S = shrimp; CI = calf intestine. All measurements done in tryplicate.

AP	Buffer	EU/µL		EU/µg enzyme		EU/mole enzyme	
		Activity	St. Dev.	Activity	St. Dev.	Activity	St. Dev.
EC-AP	0.6M Tris	2.9	0.2	0.48	0.03	4.3E+10	0.2E+10
	1.0M Tris	4.10	0.06	0.683	0.009	6.08E+10	0.08E+10
	borate	2.33	0.03	0.389	0.005	3.46E+10	0.05E+10
	bor/DMSO	1.49	0.07	0.25	0.01	2.2E+10	0.1E+10
	2A2M1P	1.60	0.03	0.266	0.005	2.37E+10	0.05E+10
HP-AP	0.6 Tris	0.054	0.003	0.028	0.002	0.36E+10	0.03E+10
	1.0 Tris	0.0875	0.0006	0.0453	0.0003	0.58E+10	0.05E+10
	borate	0.053	0.002	0.027	0.001	0.35E+10	0.03E+10
	bor/DMSO	0.0644	0.0004	0.0334	0.0002	0.43E+10	0.04E+10
	2A2M1P	0.309	0.005	0.160	0.003	2.0E+10	0.2E+10
BK-AP	0.6 Tris	0.0097	0.0001	0.0206	0.0002	3.55E+9	0.04E+9
	1.0 Tris	0.020	0.003	0.043	0.007	7E+9	1E+9
	borate	0.0166	0.0003	0.0352	0.0006	6.1E+9	0.1E+9
	bor/DMSO	0.0150	0.0003	0.0318	0.0006	5.5E+9	0.1E+9
	2A2M1P	0.36	0.07	0.10	0.02	17.8E+9	3E+9
PK-AP	0.6 Tris	0.0358	0.0007	0.0398	0.0008	6.8E+9	0.6E+9
	1.0 Tris	0.049	0.002	0.055	0.002	9.4E+9	0.8E+9
	borate	0.092	0.001	0.102	0.001	17E+9	1E+9
	bor/DMSO	0.1029	0.0009	0.114	0.001	19E+9	2E+9
	2A2M1P	0.44	0.03	0.49	0.03	84E+9	7E+9
S-AP	0.6 Tris	2.8	0.3	8.4	0.9	9E+11	1E+11
	1.0 Tris	5.6	0.1	16.3	0.4	18.0E+11	0.5E+11
	borate	4.8	0.4	14	1	15E+11	1E+11
	bor/DMSO	2.9	0.3	8.5	0.9	9E+11	1E+11
	2A2M1P	2.04	0.02	5.99	0.06	6.59E+11	0.07E+11
CI-AP	0.6 Tris	173	1	8.25	0.05	1.24E+12	0.08E+12
	1.0 Tris	1174	11	55.9	0.5	8.4E+12	0.6E+12
	borate	927	30	44	1	6.6E+12	0.4E+12
	bor/DMSO	873	32	42	2	6.2E+12	0.4E+12
	2A2M1P	6.1E+5	0.3E+5	2.9E+4	0.1E+4	4.4E+15	0.3E+15

in optical density of 1.0 per minute at 410 nm in a cuvette of 1 cm path length at pH 8.20 and 37°C. The results are expressed in units per μL of stock enzyme solution, in units per μg enzyme, and in units per mole enzyme. The calf intestinal alkaline phosphatase showed the highest molar activity in the bulk solution in all of the buffers, as expected. It was followed by the shrimp isozyme, again irrespective of the buffer. *Escherichia coli* isozyme had the third highest activity in all buffers except the high pH 2A2M1P buffer, where the porcine kidney isozyme was faster. In the borate/DMSO buffer, the one employed in single molecule on-capillary assays, the order was (from highest to lowest activity): CI-AP, S-AP, EC-AP, PK-AP, BK-AP, and HP-AP. The placental isozyme had the lowest activity in all buffers except the high pH 2A2M1P, where it was only marginally faster than BK-AP.

All alkaline phosphatase isozymes were compatible with the single molecule assay method without any adjustments. Representative sample electropherograms of each of the isozymes, all on the same scale, are shown in **Figure 3.6**. The activities of individual molecules were calculated based on the standards used with each run on any given day. Peak areas of individual molecules were estimated using Peak Fit and compared to peak areas corresponding to standard injections for a given day. Shrimp alkaline phosphatase appeared to have the highest activity in these experiments, and it is second only to CI-AP when all the isozymes assayed in the course of my research are taken into account. The distributions of activity among single molecules were rather wide - differences between the least and most active molecules varied between 13-fold for the bovine kidney isozyme and 40-fold for the human placenta alkaline phosphatase. The histograms, including the average activities and their standard deviations, for all four isozymes are shown in **Figures 3.7** and **3.8**.

The SDS-PAGE of human placenta and bovine kidney AP are shown in **Figure 3.9**. Neither isozyme appears to be homogeneous with respect to molecular weight of the subunits. The major bands are found at around 50 kDa for HP-AP and 46 kDa for

Figure 3.6 Electropherograms of representative runs done with bovine kidney (BK-AP), human placenta (HP-AP), porcine kidney (PK-AP), and shrimp alkaline phosphatases. The runs were done on 3 different days in borate/DMSO buffer. The scaling has been preserved.

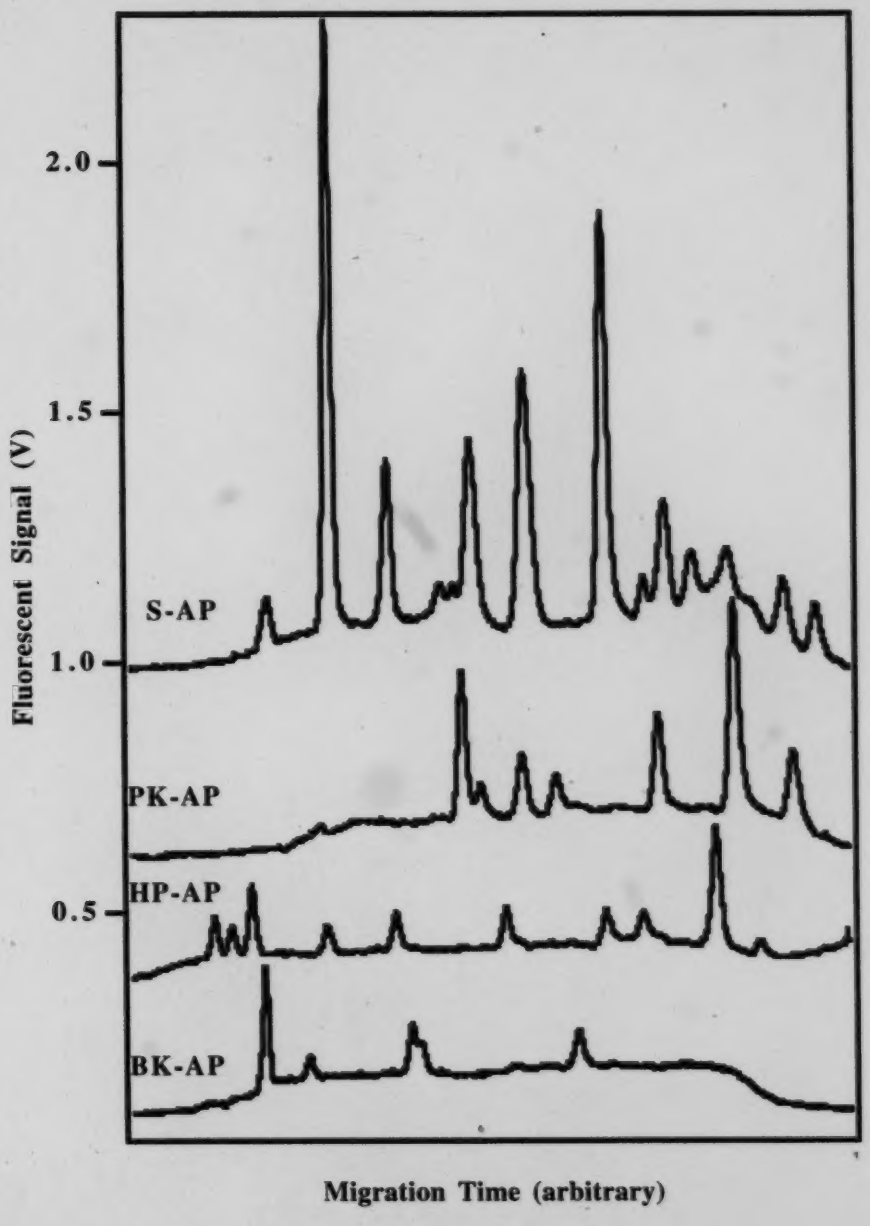
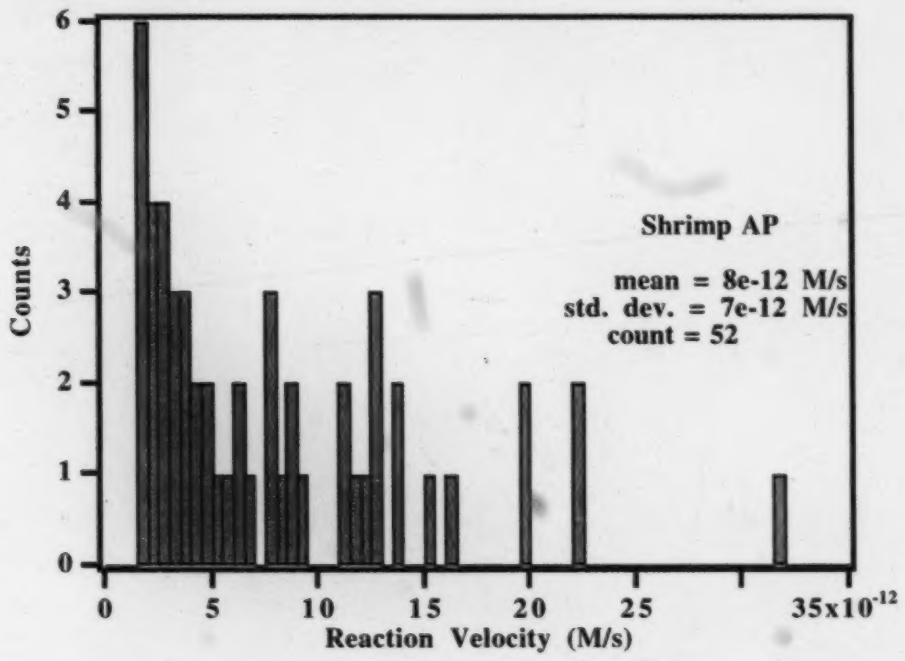


Figure 3.7 Histograms of shrimp and human placenta alkaline phosphatase single molecule activities (note the difference scales).



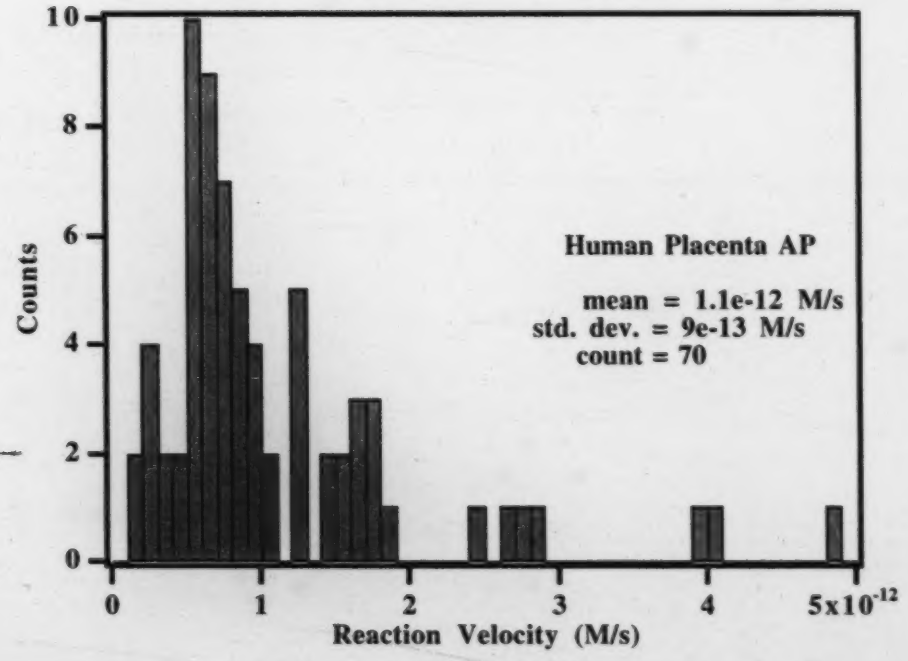


Figure 3.8 Histograms of bovine and porcine kidney alkaline phosphatase single molecule activities (note the different scales).

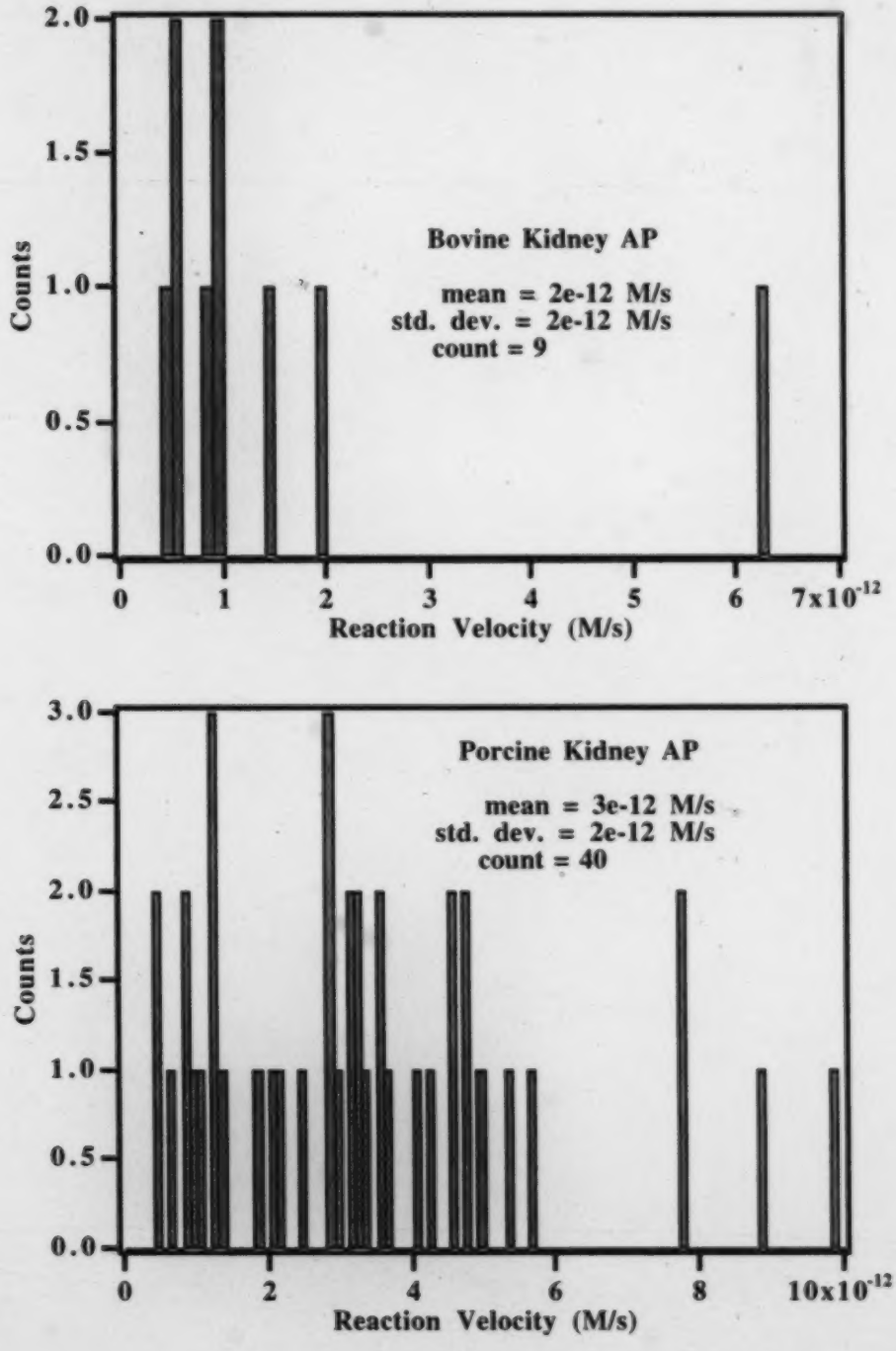
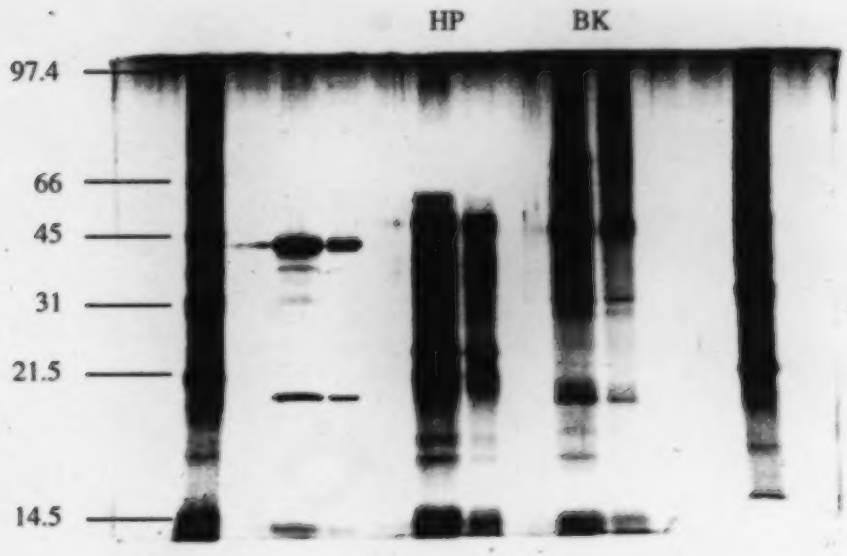


Figure 3.9 SDS-PAGE of bovine kidney (BK) and human placenta (HP) alkaline phosphatase.



BK-AP, corresponding to approximate weights of the subunits. There are, however, a number of weaker bands observable at lower molecular weights, suggesting a certain degree of proteolysis in the enzyme stocks. Sample degradation has been previously observed for the *E.coli* and calf intestinal isozymes. Isoelectric focusing of all 4 isozymes (results not shown) resulted in smearing when developed with AttoPhos™ in 1.0 M 2A2M1P at 37°C. Human placenta AP gave a long smear, stretching almost from one end of the gel to the other (a range of 3 pH units, 5-8). Porcine kidney alkaline phosphatase resulted in a tighter smear, between pH of 7 and 8. Shrimp alkaline phosphatase resulted in a tight smear in the pH 5 to 6 range. Bovine kidney AP was not observed in the gel.

3.3.4 Discussion

Of the four alkaline phosphatases assayed in this section, the shrimp isozyme appeared to have the highest activity. The lack of information about its structure however, does not support its choice as a replacement for CI-AP. Although the enzyme is known to be glycosylated, the number of carbohydrate chains, or even the number of possible attachment sites, are not known. Furthermore, the pI quoted in literature did not appear to match the pI found for this enzyme in my study. The reason for this may be the source of the enzyme. Shrimp alkaline phosphatase described in literature came from two species of shrimp: *Penaeus japonicus* and *Penaeus monod*. The two variants had different isoelectric points. The source of the commercial shrimp alkaline phosphatase is not known; it is possible that, if the enzyme is derived from a different species yet, its pI will differ from the two enzymes described in literature.

There was not much difference between the kidney isozymes. They are both good candidates for the study of glycosylation effects on activities of isoforms as they are thought to be easily deglycosylated. Based on the experience with CI-AP deglycosylation however, it is not the process of deglycosylation itself that is

problematic. Complete enzymatic removal of carbohydrates through the use of a kit requires denaturation of the enzyme which will render its useless for assays of activity. Therefore, for either of these enzymes to be useful, a method would have to be found which would allow for the removal of sugars without subsequent loss in activity. At that point, the bovine isoform would be a preferred choice because of the greater amount of information, including the amino acid sequence, available on this particular enzyme.

The human placenta isozyme was looked at not as a replacement model for CI-AP, but rather with a possibility of clinical utilization in mind. Since serum alkaline phosphatase is one of the most assayed enzymes in clinical sciences, I thought that a look at single molecules of the placental component would be of some interest. The enzyme itself is quite complex, with a number of isozymes (called types) already known, and more possible. This seems to be confirmed in the IEF study, where the placental isozyme produced a smear even larger than that seen for calf intestinal alkaline phosphatase (see **Figure 3.5 (b)**). The complexity of the enzyme is further evident in the wide distribution of the activities of single molecules - there was a 40-fold difference between the least and most active molecules.

3.4 CONCLUSIONS

Not all work in the course of a thesis research is a success. The conclusions drawn from the less-than-fabulous results reported here, however, can still be of some use. The lack of success with aminoalcohols as buffers and with isoelectric focusing of calf intestinal alkaline phosphatase prompted a change in the model enzyme under study. Since a clear link could not be drawn between the isoforms of CI-AP, a simpler and easier model, the *E. coli* isoform, was chosen. The results obtained with that isozyme validated the change in models. The broad question of enzyme structure-activity relationship was answered for that simple model and only a more specific

question of carbohydrate content's influence on activity remains. To answer this question another alkaline phosphatase needs to be found - the kidney isoforms of cow or pig are good choices for the future.

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4

The Kinetics of Single Enzyme Molecules: Studies on Immobilized Molecules of Alkaline Phosphatase

4.1 INTRODUCTION

The earlier chapters in this thesis were concerned with work done on solubilized single molecules of an enzyme. The activities of single enzyme molecules were thus measured and used in studies of structure-function relationship and thermal denaturation pathways. A limitation of these experiments however, was the fact that, at most, only a few measurements could be performed on a given molecule. Therefore, the kinetics of an enzymatic reaction could not be fully explored on a molecule by molecule basis. To attempt that feat some form of immobilization of the enzyme was necessary so that the activity of the molecule could be measured repeatedly, over a range of substrate concentrations - a Michaelis-Menten experiment on single molecules of an enzyme.

In fact, immobilization of enzymes should be considered by anybody interested in their behaviour *in vivo*. Intracellular enzymes are usually membrane-bound or solid-state assemblies and they always exist in a complex environment. Purified enzymes in dilute solutions, although certainly extremely useful in all sorts of applications, do not reflect the intricacies of enzymes *in vivo*. Therefore, to gain the insight into their work *in vivo*, attempts should be made to analyze them in either their natural environments (by single cell analysis) or to reduce the level of simplification by somehow approximating the cellular conditions. This can be at least partly achieved by immobilization.

Immobilization can be defined as "the physical confinement of enzyme molecules during a continuous physical process"(1) or as "the imprisonment of an enzyme molecule in a distinct phase that allows exchange with, but is separated from, the bulk phase in which substrate effector or inhibitor molecules are dispersed and monitored"(2). Another term, "insolubilization", is often used interchangeably in literature; it should however be avoided, since there are methods to immobilize solubilized enzymes.

There are four major categories of immobilized enzymes as recognized by the Engineering Foundation Conference (1973)[3]:

a) enzymes immobilized by physical or chemical adsorption,

b) enzymes covalently bound to soluble or insoluble polymers,

c) enzymic species arising from crosslinking of enzyme molecules,

d) entrapped enzymes.

The first class relies on ionic, hydrophobic, hydrogen bond, or Van der Waals interactions to attach a molecule to a surface. This is probably the easiest way to immobilize enzymes since it can be effected by simply bringing the enzyme in contact with the surface. The extent of adsorption will depend on such variables as pH, type of solvent, ionic strength, enzyme concentration, and temperature. The conditions need to be optimized so that maximum activity is retained. Since this is a physical method, permanent changes to the enzymes structure or function are not expected, i.e. an enzyme should regain its solubilized-form characteristics once desorbed. In its adsorbed state however, the enzyme can be a very different molecule due to factors such as conformational changes, neutralization of charges, or microenvironmental changes. The method is also reversible which can be of help when the support surface is to be reused, or a problem when the binding is not strong enough to avoid desorption of enzyme throughout the experiment. For full description of this class of immobilized enzymes, the methods used in immobilization and its applications please see Messing (1976) and Zaborsky (1973)[4,5].

The second class of immobilized enzymes is probably the most common. The methods used to achieve covalent linkage involve chemical reactions between an enzyme and a derivatized (activated) or underivatized (reactive) polymer support. A linker, or spacer, arm is often involved. On the enzyme, the groups involved in covalent bonds include terminal amino and carboxyl groups, and some surface amino acids (lysine, arginine, histidine, tryptophan, tyrosine, cysteine, serine, aspartic and

glutamic acids). The supports include agarose, cellulose, dextran, glass, starch, polypeptides, and acrylamide-, maleic anhydride-, methacrylic acid-, and styrene-based polymers. It is imperative that the covalent linkage does not involve any groups essential for activity, or ones which would induce significant changes in enzyme's structure or function once reacted. Use of spacer arms avoids large steric hindrance which may occur when the enzyme is linked directly, and hence in close proximity, to a support surface. Although the methods used require chemical reactions and so can adversely affect the enzyme, the resulting very strong bonds make covalent immobilization a very attractive option. For full description of this class of immobilized enzymes please see Methods in Enzymology, vol. 44 and Zaborsky (1973)[6-8].

The third class, cross-linked enzymes, relies on methods that involve multifunctional reagents to attach enzymes to each other (intermolecular crosslinking). In addition, these reagents also cause some intramolecular crosslinking, where amino acid residues on the same enzyme molecule can join together. The multifunctional reagents used in crosslinking include glutaraldehyde, diazobenzidine derivatives, and 1,5 - difluoro - 2,4 - dinitrobenzene. The resulting enzymic species can range from water soluble oligomeric species to highly insoluble enzyme polymers in three dimensional networks. The control of the extent of intra- vs. intermolecular crosslinking is difficult to achieve and inactivation of enzyme can also be a problem (unless the active site can be blocked reversibly during crosslinking). For full description of this class of enzymes please see Broun (1976), Zaborsky (1973), and Martinek (1988)[9-11].

The final class of immobilized enzymes relies on entrapment methods including: - highly crosslinked polymers - such as polyacrylamide gels, starch gels, or glass sol gels, where the size of the pores can be adjusted to the enzyme's molecular weight (size)[12,13],

- microencapsulation within semipermeable capsules which can be either permanent (cellulose nitrate, polystyrene, ethylcellulose, polyamide) or nonpermanent (a liquid membrane composed of hydrocarbon solvent, surfactants, and additives enclosing aqueous droplets of enzyme and reagents)[14-16],

- containment within semipermeable membranes, either synthetic (ultrafiltration and dialysis membranes) or natural (reconstituted membrane-protein systems)[17,18],

- optical entrapment where lasers are used to fix enzymes in space while reagents are flowed through the reaction cell.

The first two methods have the advantage of not restricting the enzyme's movements, effectively leaving the enzyme in a solubilized state. Natural semipermeable membranes are excellent at mimicking the *in vivo* environment for those enzymes that naturally occur in membranes (such as membrane transport systems). Optical entrapment is relatively new; it allows the study of enzymes in gaseous phase.

It is very important to point out that immobilization does not mean a complete loss of freedom of movement for the molecule. The methods described above afford to the enzyme varying degrees of freedom to move - from a multi-point covalent attachment, resulting in a total loss of freedom and possibly activity, to entrapment within a gel or a microcapsule, leaving the molecule free to move, yet still separated from the bulk solution.

In any case, immobilization may affect the enzyme's intrinsic properties as well as its microenvironment. Depending on the immobilization method a number of changes can occur in the enzyme molecule. It can change its conformation, only slightly or drastically, to a point where it may denature. It may lose surface charges. The active site may become blocked either by a reagent used in immobilization or through spatial hindrance. Catalytically essential amino acids can be modified. The environment around the enzyme also changes. Charged surfaces may attract oppositely charged ions creating a concentration gradient between the surface and bulk solution.

In case of hydronium ion, this will lead to pH variations between the bulk solution and the enzyme's surroundings. Steric hindrance may be encountered at the surface due to surface modifications, or the molecular size of the polymer which makes up the surface, or the orientation of the attached enzyme. The thickness of the diffusion layer around the immobilized enzyme may vary with the type of support. Variations can also occur in hydrophilicity of and electrostatic interactions at the surface. These changes, on their own or in any combination, can lead to changes in activity, pH dependence, kinetic constants, specificity and stability of the enzyme as compared to pure enzyme in dilute solution. The relationships are very complex and for a thorough review of the subject the reader should consult Zaborsky (1973)[1].

Immobilized enzymes have found their use in many disciplines: chemistry, biochemistry, and pharmaceutical, medical, and food industries. Aside from studies of enzyme properties[19-21], investigations have been carried out in such areas as analytical chemistry, protein synthesis, protein sequencing, enzyme analog synthesis, fuel cells, organic synthesis[22,23], milk, sugar, and alcohol production, and various clinical uses.

In the field of single molecule enzymology immobilization has also found its use. Edman *et al.* (1999) immobilized biotinylated horseradish peroxidase on glass cover slips covalently coated with streptavidin for studies of single enzyme molecule turnovers by fluorescence spectroscopy[24]. Lu *et al.* (1998) used agarose gels to trap single molecules of cholesterol oxidase in their studies of enzymatic turnovers[25]. Single molecules of lactate dehydrogenase were trapped inside membranes and silica nanovials by Tan and Yeung (1997) who measured their real-time and averaged reaction rates[26]. Gelles and colleagues (1991-1998) worked with immobilized RNA polymerase[27-30]. Ha *et al.* (1999) used fluorescence spectroscopy to probe conformational dynamics and cleavage mechanism of Staphylococcal nuclease immobilized on glass coverslips via hexahistidine tags[31].

Immobilized alkaline phosphatase of *E. coli* has previously been characterized by Shan *et al.*[32] who used CNBr-activated Sepharose 4B as a support for a covalent attachment of the enzyme. The results obtained for immobilized enzyme packed-bed reactor (IEPBR) using flow injection analysis (FIA) showed for the immobilized alkaline phosphatase a reduction in product inhibition and an increase in K_m value as compared with free enzyme. The optimum pH for the immobilized enzyme was almost identical to that for the free enzyme as was the optimal buffer composition. Other studies on immobilized alkaline phosphatases were performed by Zingaro and Uziel[33] (on AP covalently bound to various polymers), van Duijn *et al.*[34] (on AP trapped within a polyacrylamide gel), Rony[35] (on AP trapped in hollow tubes), and Cyboron and Wuthier[36] (on membrane bound chicken epiphyseal cartilage AP). McCracken and Meighen (1987) studied subunits of alkaline phosphatase covalently immobilized on Sepharose CL-4B support[37,38]. All of these studies were done on bulk immobilized enzyme.

For the study of immobilized single molecules of alkaline phosphatase I chose a covalent linkage to a modified fused silica surface through a spacer arm. This was the most promising approach when coupled with capillary electrophoresis, providing good stability of the linkage while allowing to maintain the experimental set-up and conditions used in free single molecule studies.

4.2 THE IMMOBILIZATION OF ACTIVE ALKALINE PHOSPHATASE MOLECULES ON MODIFIED SILICA SURFACE

The objective of the immobilization was to attach a small number of active alkaline phosphatase molecules onto the surface of a bare silica capillary. It was important that the coverage density be low, and that the molecules be attached through a spacer arm, so as to allow for a relatively unhindered access of substrate to the enzyme. This was supposed to minimize the changes in the kinetics of the molecules due to their

restricted motion. The method chosen for the immobilization of active alkaline phosphatase molecules had been originally adapted from Bhatia *et al.*[39] and then used in our laboratory by Ahmadzadeh[40] for the immobilization of antibodies onto silica surfaces.

The method was further adapted in an attempt to gain some control over the length of the immobilized enzyme plug. It was important to be able to place a rather short plug of immobilized enzyme within the region of the capillary enclosed inside the heater unit, so that temperature during the kinetic measurements could be monitored. Having a short plug would also allow for the entire capillary to be relatively short, thus reducing the time each experiment would take. Since the immobilization method relies on consecutive injections of various reagent solutions, the times necessary for these solutions to travel the length of the capillary were measured. Consequently, estimates were made of the times needed to fill an appropriate length of the capillary with a given solution.

4.2.1 Reagents

For coating of a 10 μm i.d. capillary, all the reagents had to be free of any suspended particles. Since some of the reagents used were organic solvents, which were incompatible with our filtration system, the highest grade solvents were purchased. All toluene solutions were prepared in, and injected out of, glass vials to avoid contamination by plastics. The N_2 gas was pre-purified.

Fused silica capillaries (10 μm i.d., 150 μm o.d.) were purchased from Polymicro Technologies (Phoenix, AZ). Thick-walled, 5 mL glass reaction vials with a cone bottom (Reacti-Vial™) were obtained from Pierce (Rockford, IL). The brass bomb was machined locally to fit the reaction vial. Toluene, dimethyl sulfoxide (DMSO), and NaOH were all purchased from Sigma (Oakville, ON).

3-Mercaptopropyltrimethoxysilane (MTS) and N-succinimidyl-4-maleimidobutyrate (GMBS) were purchased from Fluka Chemie AG (Switzerland).

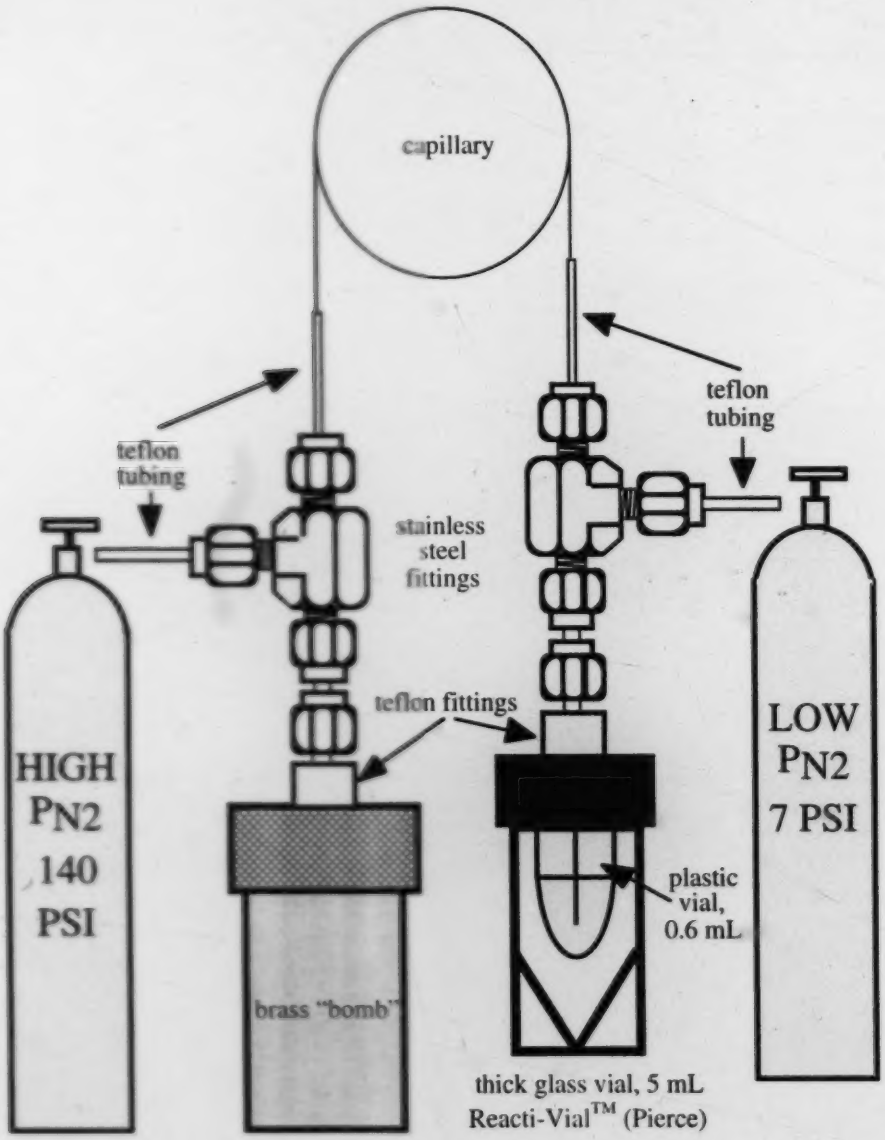
4.2.2 Procedure for the Immobilization of Single Enzyme Molecules

A fused silica capillary: 10 μm i.d., 150 μm o.d., approximately 80 cm long, was mounted, with the use of appropriate fittings, between two pressurized glass sample vials. The vials were connected to two N_2 tanks, one with a high pressure regulator and the other with a low pressure one. The low pressure end was used for introducing solutions when accurate control of the plug's length was to be achieved. The high pressure end was used for flushing out the capillary and the introduction of solutions to cover the entire capillary. The glass vial at the high pressure end was contained within a brass bomb to prevent it from exploding if the pressure became too high. For schematic representation of this set-up, please see **Fig. 4.1**.

The capillary was initially conditioned with NaOH by flushing in a 100 mM solution at high pressure for approximately 60 min. This was followed with a high pressure flush with deionized water for 15 min. The capillary was then left overnight at room temperature.

Next, the capillary was filled with toluene for 30 min. at 140 psi. The wall was modified using a 4% MTS solution in toluene. The solution was introduced at 14 psi for 3 minutes to cover an estimated 10 cm of the capillary. A plug of toluene was then introduced at 14 psi for 3 min. to push the MTS plug a further 10 cm into the capillary. The capillary was incubated with MTS for one hour at room temperature. MTS/toluene solution was then replaced with 4% DMSO in EtOH at 140 psi. This solution was introduced from the high pressure (i.e. detection) end of the capillary so that the MTS would not sweep over the untreated parts of the capillary. Next, the crosslinker

Figure 4.1 A schematic diagram of the set-up used for the immobilization of enzyme molecules on capillary. The high-pressure brass bomb (shown on the left) contains within it another glass reaction vial (as shown on the right). The diagram is not to scale.



(GMBS) was introduced as a 2 mM solution in DMSO/EtOH. This was done at 14 psi for 6 min. to cover an estimated 10 cm. A 10 cm plug of DMSO/EtOH was then introduced at 14 psi for 6 min. The capillary was again incubated for one hour at room temperature. After the incubation, the capillary was flushed out with 100 mM NaCl at 140 psi for 30 min. Finally, an appropriate dilution of alkaline phosphatase in aqueous buffer (see **Section 4.3**) was introduced at 14 psi for 10 min. to create an estimated 20 cm plug of enzyme. The capillary was once again incubated for one hour at room temperature. The enzyme solution was flushed out, from the high pressure end, with 100 mM borate, pH 9.5, to allow for hydrolyzation of unoccupied crosslinker sites. The capillary was then stored in a vial of appropriate buffer at 4°C, or, if it were to be used immediately, it was allowed one hour incubation time. A schematic diagram of this procedure is shown in **Figure 4.2**. The chemistry of the capillary wall derivatization and enzyme binding is shown in **Figure 4.3**.

4.2.3 A ZERO DEAD-VOLUME FUSED SILICA CAPILLARY REACTOR FOR STUDIES OF IMMOBILIZED ENZYME MOLECULES

The procedure described above produced a seamless fused silica reactor having a zero dead-volume. Starting from the injection end, the first 10 cm of the capillary remained bare (although the enzyme was incubated within that volume, the walls should not have been derivatized). The enzyme was confined to the next 10 centimeters, a region where the temperature could be controlled with the Peltier heater/cooler unit. Finally, the remainder of the capillary was underivatized to allow for the separation of the analyte. The capillary could be made as long as needed or cut to any desired length beyond 30 cm. Both ends of the capillary had the polyimide removed.

Figure 4.2 A schematic diagram of the immobilization procedure.

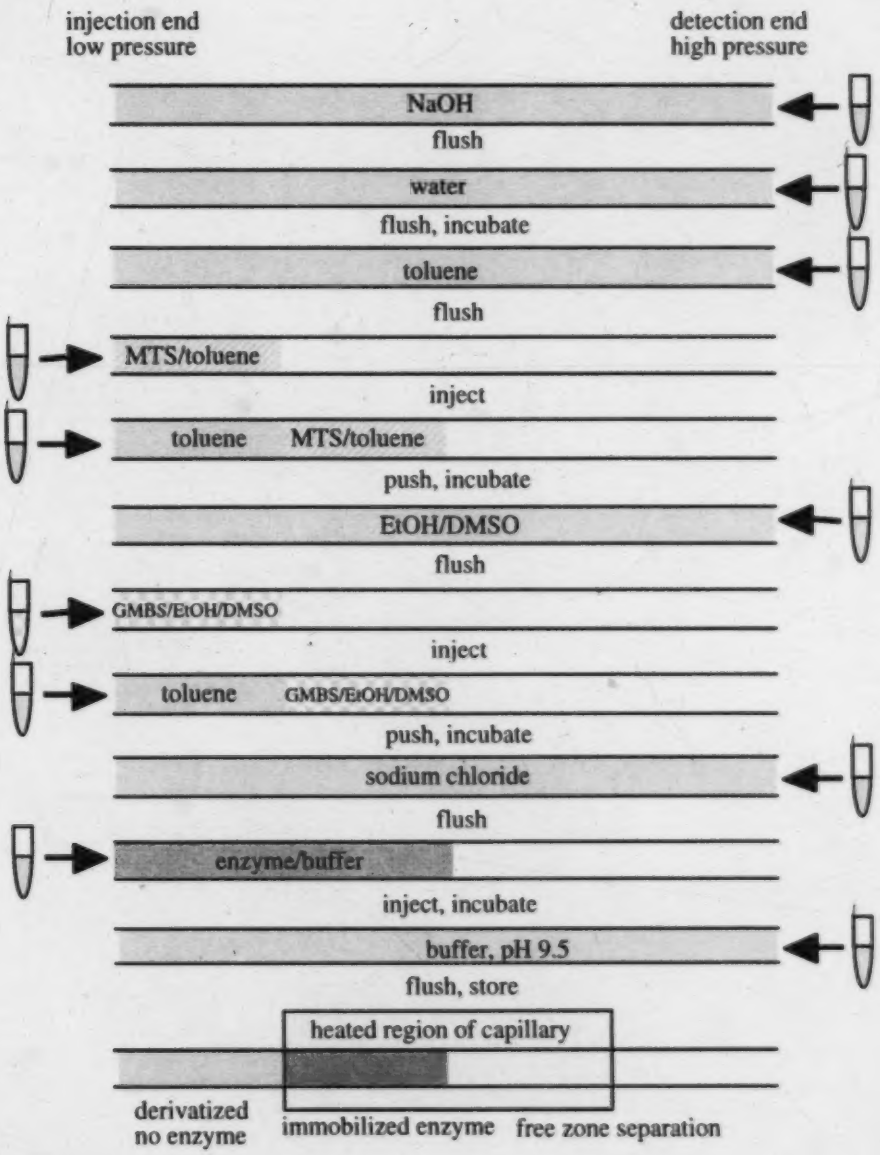
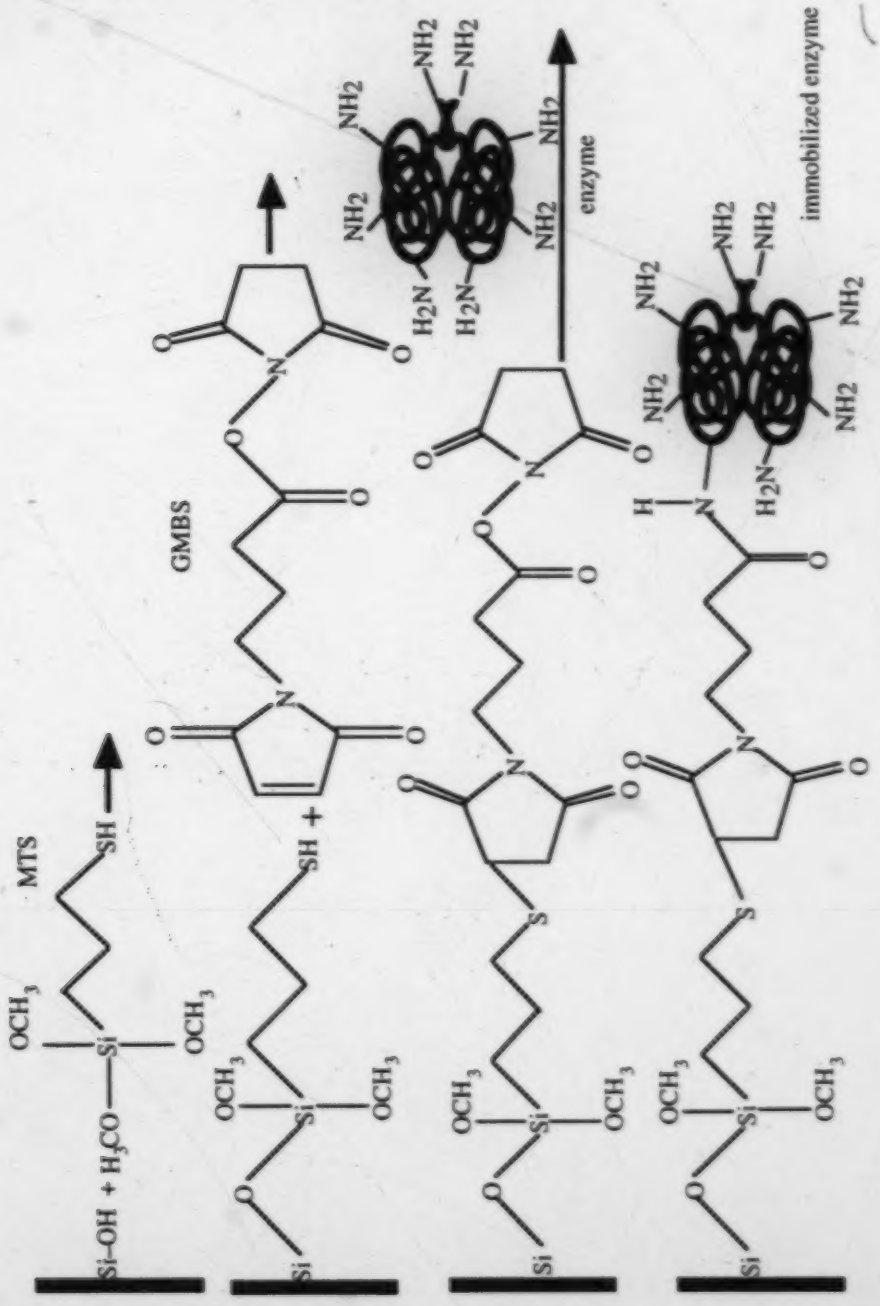


Figure 4.3 The schematic of the chemistry of capillary wall derivatization and enzyme binding (note: the drawing is not to scale).



4.3 KINETICS OF SINGLE ENZYME MOLECULE REACTIONS

With the enzyme molecules bound to the capillary wall through the MTS linker arm, a look at the kinetics of single enzyme molecules could be attempted. Molecules of alkaline phosphatase from *E. coli*, calf intestine, and porcine kidney were immobilized in different capillaries. Experiments were performed on *E. coli* and calf intestinal enzyme.

Experimental conditions had to be modified slightly from the ones employed in standard measurements of single molecule activity (see **Section 2.4.1**). There was no longer any need to inject enzyme with the substrate. Because only a few immobilized molecules would be monitored at the time, the plug of substrate could be a lot shorter and therefore the capillary could be made much shorter to allow for faster experiments. Finally, three standards were used in each run to provide better calibration curves.

4.3.1 STUDIES ON IMMOBILIZED MOLECULES OF *E. COLI* ALKALINE PHOSPHATASE

The enzyme used for immobilization was the same as Preparation 2 from **Section 2.2**. A $1/10^9$ dilution of stock enzyme in 100 mM NaCl was injected onto the capillary and incubated for 65 min. After immobilization the capillary was stored in 100 mM Tris, pH 8.2, at 4°C. Before installing in the fluorescence instrument the capillary was cut to a final length of 64 cm.

Only six runs were performed with the bacterial enzyme due to a break in the capillary. In all of them, 1.0 mM AttoPhos™ in sample buffer (86.5 mM borate/ 13.5% DMSO, see **Section 2.3.2**) was injected at 400 V/cm for a time of 120, 180, 300, or 360 s. Running buffer (85 mM borate/15% DMSO) was then injected at 400 V/cm for 120 s (240 s in the case of 120 s injection). The substrate was incubated on capillary for 20, 30, or 60 min. Finally, running buffer was electrophoresed at 400

V/cm to sweep the substrate and product past the detector. The resulting electropherograms are shown in **Figure 4.4**.

As can be seen in **Figure 4.4**, only one molecule appeared common to all of the electropherograms. Since the standards were not run with the substrate injection, the activity of this molecule cannot be measured. The peak areas however, were estimated using PeakFit and they showed a linear relationship with the incubation time, as shown in **Figure 4.5**. Since the peak area is measured above the baseline, the area for incubation time of 0 min. is, by definition, zero. The (0,0) point was thus included in the analysis.

The activity of this molecule appeared rather low compared to the activities of free enzyme molecules under otherwise identical assay conditions. Therefore I decided to use the calf intestinal enzyme for further studies on immobilized single enzyme molecules. Nonetheless, these runs represent our first successful immobilization of a single enzyme molecule on a capillary, which was later followed by repeated measurements of its activity.

4.3.2 STUDIES ON IMMOBILIZED MOLECULES OF CALF INTESTINAL ALKALINE PHOSPHATASE

The bulk of the data for the studies of the kinetics of single enzyme molecules was obtained using immobilized calf intestinal alkaline phosphatase. As previously mentioned, this enzyme is much more active than the bacterial counterpart. Of the 10 capillaries with immobilized calf intestinal alkaline phosphatase only 2 were successfully used for more than 2 days. The discussion of the kinetics of single molecules of calf intestinal alkaline phosphatase will therefore be based on the results obtained from those two capillaries.

Figure 4.4 Electropherograms of the first immobilized alkaline phosphatase molecule. The enzyme came from *E. coli* Preparation 2. Only one molecule was captured in this case. The activity of this molecule was relatively low. All assays were performed in the same concentration of substrate but for varying times (20, 20, 30, 60, 30, 40 min. starting from the bottom trace).

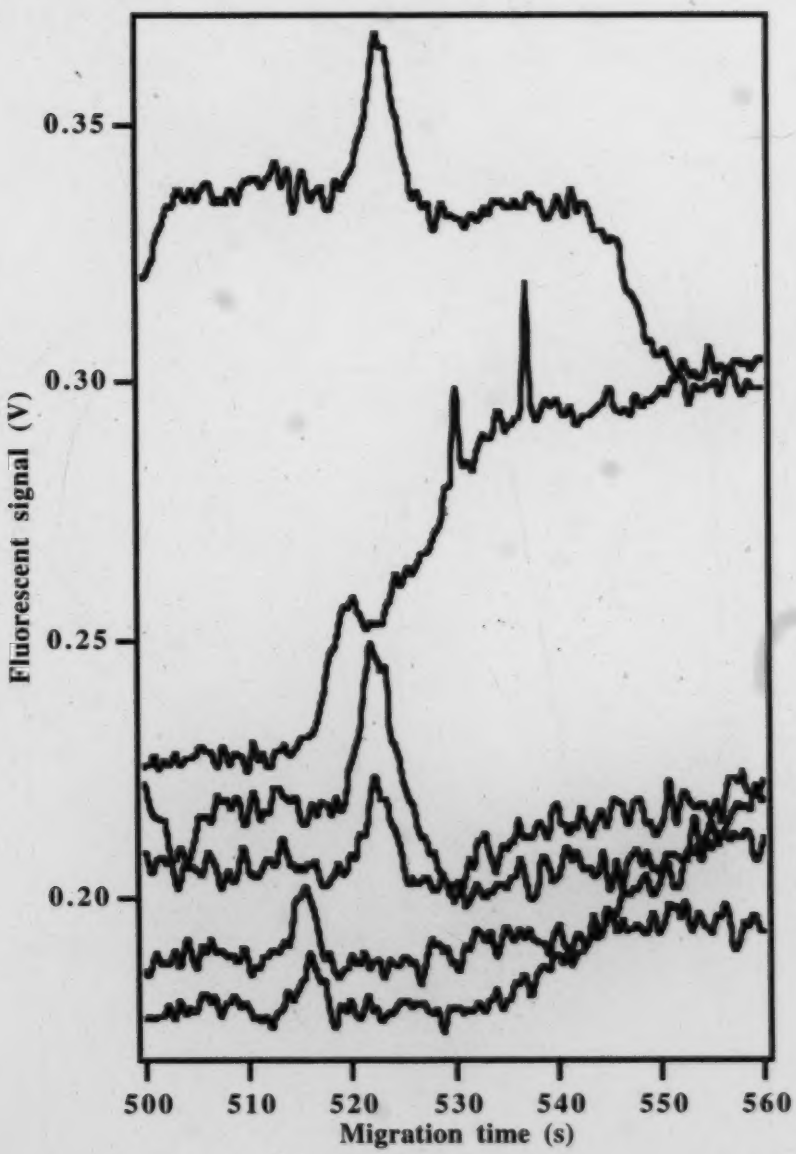
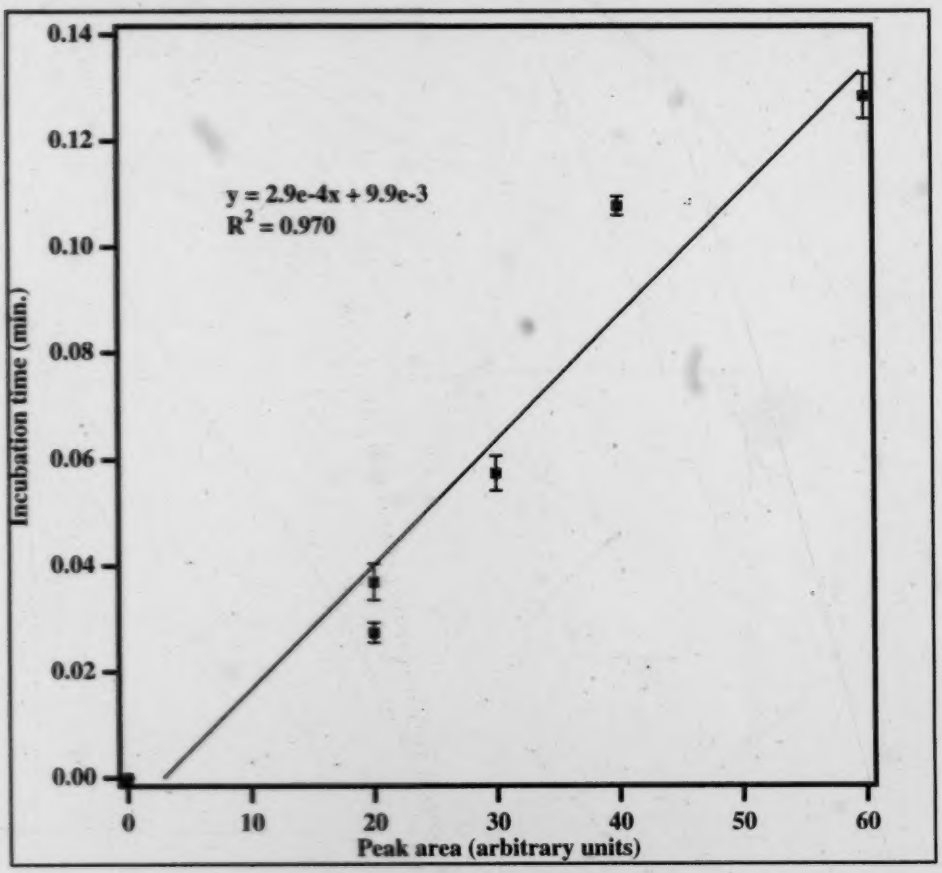


Figure 4.5 Peak areas for replicate incubations of a single immobilized molecule of *E. coli* alkaline phosphatase.



Point	incubation_time	peak_area	std_dev
0	0	0.000	0.000
1	20	0.027	0.002
2	20	0.037	0.003
3	30	0.057	0.003
4	40	0.108	0.002
5	60	0.128	0.004

4.3.2.1 Reagents

The enzyme used for immobilization was calf intestinal alkaline phosphatase purchased from Gibco BRL (Life Technologies, Burlington, ON). The enzyme comes in 50% glycerol (v/v) buffered with 25 mM Tris-HCl (pH 7.6), 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$. The specific activity of this preparation was given to be 25 units/μL, or 2,000 units/mg, where 1 unit is the amount of enzyme that will hydrolyze 1 μmol p-NPP per minute at 37°C in 1 M DEA buffer containing 0.25 mM $MgCl_2$ and 10 mM substrate, pH 9.8. The calculated enzyme mass concentration is then 12.5 mg/mL and the concentration, assuming molecular weight of 140 kDa, is 9×10^{-5} M in the stock solution.

4.3.2.2 Immobilization of enzyme molecules

The procedure for immobilization of the enzyme molecules followed the general method described in **Section 4.2.2**. The enzyme dilution used for immobilization was approximately a billion-fold from the stock. Two capillaries were deemed useful for kinetic analysis: capillary nos. CI-13 and CI-18.

4.3.2.3 CE conditions

The assay protocol varied vary slightly between the two capillaries. Both protocols are described below with the variables given in **Tables 4.1** and **4.2** for capillaries nos. 13 and 18, respectively. The buffers used in these experiments were the same as the ones described in **Section 2.4.1**:

running buffer = 85 mM borate/ 15% DMSO, pH 9.5

sample buffer = 86.5 mM borate/ 13.5 % DMSO, pH 9.5

(standards and sample are prepared in sample buffer)

sheath-flow buffer = 100 mM borate, pH 9.5

The instrument has been previously described in **Section 2.2.3**. Capillaries nos. CI-13 and CI-18 were cut to a final length of 60 cm and 44.5 cm, respectively.

The CE protocol for capillary no. CI-13 was as follows:

1) inject 1st standard (low concentration) at 80 V/cm for 10 seconds;

2) electrophorese running buffer at 400 V/cm for 20 seconds;

3) inject 2nd standard (middle concentration) at 80 V/cm for 10 seconds;

4) electrophorese running buffer at 400 V/cm for 20 or 40 seconds;

5) inject 3rd standard (high concentration) at 80 V/cm for 10 seconds;

6) electrophorese running buffer at 400 V/cm for 80 seconds;

7) inject substrate (AttoPhos™) at 400 V/cm for $t_{injection}$

8) electrophorese running buffer at 400 V/cm for t_{push}

9) incubate for $t_{incubation}$

10) electrophorese running buffer at 400 V/cm

The substrate concentration range employed was 0.1 - 4.0 mM. Most concentrations were done only once.

The CE protocol for capillary no. CI-18 was as follows:

1) inject 1st standard (low concentration) at 100 V/cm for 10 seconds;

2) electrophorese running buffer at 500 V/cm for 20 seconds;

3) inject 2nd standard (middle concentration) at 100 V/cm for 10 seconds;

4) electrophorese running buffer at 500 V/cm for 20 seconds;

5) inject 3rd standard (high concentration) at 100 V/cm for 10 seconds;

6) electrophorese running buffer at 500 V/cm for 60 seconds;

7) inject substrate (AttoPhos™) at 500 V/cm for $t_{injection}$;

8) incubate for $t_{incubation}$;

9) electrophorese running buffer at 500 V/cm;

The substrate concentration range was expanded to 1 μM - 7.5 mM. Most concentrations were repeated in triplicate.

Table 4.1 Run conditions for kinetic assays of immobilized calf intestinal alkaline phosphatase on capillary no. CI-13.

Run	Date	[AttoPhos™] (mM)	$t_{injection}$ (sec)	t_{push} (sec)	$t_{incubation}$ (sec)	standards [FE] (M)
1	05/06/00	1.0	360	120	900	none
2	05/06/00	1.0	360	120	600	none
3	05/06/00	1.0	300	0	600	none
4	06/06/00	1.0	360	120	900	none
5	06/06/00	1.0	390	90	900	$4, 8, 16 \times 10^{-9}$
6	06/06/00	2.0	390	90	975	$4, 8, 16 \times 10^{-9}$
7	06/06/00	3.0	390	90	900	$4, 8, 16 \times 10^{-9}$
8	06/06/00	4.0	390	270	900	$4, 8 \times 10^{-9}$
9	06/06/00	4.0	390	90	900	$4, 8, 16 \times 10^{-9}$
10	07/06/00	1.0	390	90	900	$4, 8, 16 \times 10^{-9}$
11	07/06/00	0.8	390	90	900	4×10^{-9}
12	07/06/00	0.6	390	90	900	$4, 8, 16 \times 10^{-9}$
13	07/06/00	0.4	390	90	900	$2, 4, 8 \times 10^{-9}$
14	07/06/00	0.2	390	90	900	$2, 4, 8 \times 10^{-9}$
15	07/06/00	0.3	390	99	900	$2, 4, 8 \times 10^{-9}$
16	07/06/00	0.1	390	90	900	$2, 4, 8 \times 10^{-9}$
17	08/06/00	1.0	390	90	900	$2, 4, 8 \times 10^{-9}$
18	08/06/00	0.5	390	90	900	$2, 4, 8 \times 10^{-9}$
19	08/06/00	1.5	360	120	960	$2, 4, 8 \times 10^{-9}$
20	08/06/00	2.5	330	150	540	$2, 4, 8 \times 10^{-9}$
21	08/06/00	3.5	330	150	900	$2, 4, 8 \times 10^{-9}$

Table 4.2 Run conditions for kinetic assays of immobilized calf intestinal alkaline phosphatase on capillary no. CI-18.

Run	Date	[AttoPhos™] (mM)	$t_{injection}$ (s)	$t_{incubation}$ (s)	Standards [FE](M)	Peaks detected
1	12/07/00	1.0	120	900	none	not analyzed
2	12/07/00	1.0	60	900	none	not analyzed
3	12/07/00	1.0	120	900	none	not analyzed
4	12/07/00	1.0	180	900	none	not analyzed
5	12/07/00	1.0	300	900	none	not analyzed
6	13/07/00	1.0	120	905	none	P1-P6
7	13/07/00	1.0	120	920	$1, 5, 10 \times 10^{-8}$	P1-P6
8	13/07/00	1.5	150	910	$1, 3, 5 \times 10^{-8}$	P1-P6
9	13/07/00	0.5	60	900	$1, 3, 5 \times 10^{-8}$	P1-P6
10	13/07/00	0.1	60	930	$1, 3, 5 \times 10^{-8}$	P1-P6
11	13/07/00	0.1	60	2700	$1, 3, 5 \times 10^{-8}$	P1-P6
12	13/07/00	0.7	60	900	$1, 3, 5 \times 10^{-8}$	P1-P6
13	13/07/00	0.1	60	1800	$1, 3, 5 \times 10^{-8}$	P1-P6
14	13/07/00	2.0	60	600	$1, 3, 5 \times 10^{-8}$	P1-P6
15	13/07/00	0.3	60	900	$1, 3 \times 10^{-8}$	P1-P6
16	13/07/00	0.5	60	610	$1, 3, 5 \times 10^{-8}$	P1-P6
17	14/07/00	1.2	60	900	$1, 3, 5 \times 10^{-8}$	P1-P6
18	14/07/00	1.2	60	600	$1, 3, 5 \times 10^{-8}$	P1-P6
19	14/07/00	1.7	60	600	$1, 3, 5 \times 10^{-8}$	P1-P6
20	14/07/00	1.7	60	900	$1, 3, 5 \times 10^{-8}$	P1-P6
21	14/07/00	0.05	60	1800	$1, 3, 5 \times 10^{-8}$	P1-P6
22	14/07/00	0.05	60	1200	$1, 3, 5 \times 10^{-8}$	P1-P6
23	14/07/00	0.9	60	300	$1, 3, 5 \times 10^{-8}$	P1-P6
24	14/07/00	0.9	60	900	$1, 3, 5 \times 10^{-8}$	P1-P6
25	14/07/00	2.5	60	300	$1, 3, 5 \times 10^{-8}$	P1-P6
26	14/07/00	3.5	60	300	$1, 3, 5 \times 10^{-8}$	P1-P6
27	14/07/00	3.5	60	330	$1, 3, 5 \times 10^{-8}$	P1-P6
28	17/07/00	0.9	60	600	none	P1-P6
29	17/07/00	0.7	60	600	$1, 3, 5 \times 10^{-8}$	P1-P6
30	17/07/00	0.7	60	300	$1, 3, 5 \times 10^{-8}$	P1-P6
31	17/07/00	0.3	60	600	$1, 3, 5 \times 10^{-8}$	P1-P6
32	17/07/00	0.3	60	1230	$1, 3, 5 \times 10^{-8}$	P1-P6
33	17/07/00	0.05	60	600	$1, 3, 5 \times 10^{-8}$	P1-P6

Table 4.2 continued from previous page

Run	Date	[AttoPhos™] (mM)	$t_{injection}$ (s)	$t_{incubation}$ (s)	Standards [FE] (M)	Peaks detected
34	17/07/00	0.5	60	300	$1, 3, 5 \times 10^{-8}$	P1-P6
35	17/07/00	1.5	85	600	$1, 3, 5 \times 10^{-8}$	P1-P6
36	17/07/00	1.5	60	300	$1, 3, 5 \times 10^{-8}$	P1-P6
37	17/07/00	2.0	60	900	$1, 3 \times 10^{-8}$	P1-P6
38	17/07/00	1.0	60	630	$1, 3, 5 \times 10^{-8}$	P1-P6
39	17/07/00	1.2	60	300	$1, 3, 5 \times 10^{-8}$	P1-P6
40	18/07/00	2.5	60	900	none	P1-P6
41	18/07/00	2.5	60	620	not analyzed	not analyzed
42	18/07/00	3.0	60	900	$1, 3, 5 \times 10^{-8}$	P1-P6
43	18/07/00	3.0	60	600	$1, 3, 5 \times 10^{-8}$	P1-P6
44	18/07/00	1.7	60	300	$1, 3, 5 \times 10^{-8}$	P1-P6
45	18/07/00	2.0	60	300	$1, 3, 5 \times 10^{-8}$	P1-P6
46	18/07/00	3.5	60	900	$1, 3, 5 \times 10^{-8}$	P1-P6
47	18/07/00	3.5	60	600	$1, 3, 5 \times 10^{-8}$	P1-P6
48	18/07/00	0.01	60	600	not analyzed	P3, P5, P6
49	18/07/00	0.01	60	1200	none	P3, P5, P6
50	18/07/00	0.01	60	1800	$1, 3, 5 \times 10^{-9}$	not analyzed
51	18/07/00	0.005	60	600	$1, 3, 5 \times 10^{-9}$	P5
52	19/07/00	0.001	60	1200	none	not analyzed
53	19/07/00	0.005	60	1800	$1, 3, 5 \times 10^{-9}$	P3, P5, P6
54	19/07/00	0.005	60	1200	$1, 3, 5 \times 10^{-9}$	P3, P5, P6
55	19/07/00	4.0	60	300	$1, 3, 5 \times 10^{-8}$	not analyzed
56	19/07/00	4.0	60	960	$1, 5 \times 10^{-8}$	P1-P6
57	19/07/00	4.5	60	300	$1, 3, 5 \times 10^{-8}$	P1-P6
58	19/07/00	5.0	60	310	$1, 3, 5 \times 10^{-8}$	P1-P6
59	19/07/00	5.5	60	300	$1, 3, 5 \times 10^{-8}$	P1, P4-P6
60	20/07/00	6.0	60	330	$1, 3, 5 \times 10^{-9}$	not analyzed
61	20/07/00	6.5	60	900	$1, 3, 5 \times 10^{-8}$	not analyzed
62	20/07/00	7.0	60	900	$1, 3, 5 \times 10^{-8}$	not analyzed
63	20/07/00	7.5	60	600	$1, 3, 5 \times 10^{-8}$	not analyzed

In case of runs when standards were not used, the protocols started at the 7th step and proceeded as above. The assays were also run in overlap, i.e. the next run was started before the preceding run was finished, in order to save time. The temperature of the assays was 40 ± 0.1°C. For overnight and over-the-weekend storage, the capillaries were stored in 100 mM borate, pH 9.5, at 4°C by means of the same Peltier heater/cooler unit as used for controlling assay temperature. At least one hour of temperature equilibration was allowed before the first enzyme assay of the day.

4.3.2.4 Bulk kinetic assay of free calf intestinal alkaline phosphatase

In order to compare the results of kinetic assays on immobilized single molecules of calf intestinal alkaline phosphatase, a measurement of kinetics of a bulk sample was performed. This was done on free enzyme sample so as to avoid a large uncertainty in enzyme concentration associated with enzyme immobilization. This uncertainty is not an issue in single molecule experiments where the number of molecules is counted. Aside from the solubilization state of the enzyme and its concentration, all other conditions were kept the same.

The kinetics of the free enzyme were measured over an AttoPhos™ concentration range of 1.0×10^{-6} M to 5.5×10^{-3} M in 86.5 mM borate, 13.5% DMSO, pH 9.5, at 40°C using SpectraMax Gemini XS fluorescence plate reader from Molecular Devices (Sunnyvale, CA) and FluoroNunc Black Polysorb fluorescence microplates from VWR Canlab (Mississauga, ON). The excitation wavelength was 458 nm with emission at 580 nm; PMT was on medium setting. Samples were prepared and run in triplicate with a reading taken every minute for 15 minutes, after mixing. Enzyme was at $1/7 \times 10^{7}$ dilution from stock commercial preparation. AttoPhos™, at a given concentration, was used as a blank for each set of samples. A calibration curve was generated using Fluorescent Emitter concentrations of 7×10^{-8} M, 1×10^{-7} M, $3 \times$

10^{-7} M, 7×10^{-7} M, 1×10^{-6} M, 3×10^{-6} M, 7×10^{-6} M, and 1×10^{-5} M. The raw data, in Relative Fluorescence Units (RFUs), was analyzed in IgorPro.

Enzyme concentration was measured using Bio-Rad Protein Assay (Bio-Rad, Hercules, CA), a method based on the Bradford procedure, on the V_{max} Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA). Measurements were made in microtiter plates, at 595 nm, at RT, using IgG to standardize the readings. Eleven samples were measured at a dilution of 1/50 from stock commercial preparation.

4.3.2.5 Results of the kinetic assays on immobilized single molecules of calf intestinal alkaline phosphatase

The first kinetic study of immobilized single calf intestinal alkaline phosphatase molecules was completed using capillary no. CI-13. The electropherograms obtained from capillary no. CI-13 are shown in **Figures 4.6** and **4.7**. The capillary contained a large number of alkaline phosphatase molecules of which at least one persisted throughout all the runs. Six more molecules from that capillary were found to be useful in the analysis. The molecules were chosen for the analysis on the condition that their product peaks were well resolved. The peaks which were to be analyzed were designated CP (for the central peak, marked with an arrow in **Figures 4.6** and **4.7**), and P1-6 (for peaks 1 - 6, to the right of the central peak). **Table 4.3** shows the presence of these peaks in the 20 runs (including the first day's runs, which were not used in analysis). There appeared to be more molecules which were persistent over a useful range of substrate concentrations; most of them were, however, badly resolved. The capillary lasted for 21 runs over 4 days. The first 3 runs were not used in analysis because there were no standards for the first day (see **Table 4.1**). Run no. 8 was also not analyzed due to a mistake in assay protocol which invalidated the data obtained. This run is not included in **Figures 4.6** and **4.7**, nor in **Table 4.3**.

Figure 4.6 A set of 20 electropherograms obtained for capillary no. CI-13. The peak shown with the arrow is consistent for all runs. Six more peaks were selected for analysis; they persisted in the capillary for between 10 and 19 runs. The electropherograms show some drift in migration times of the product peaks; they are shown with the first run at the bottom; run 8 is omitted.

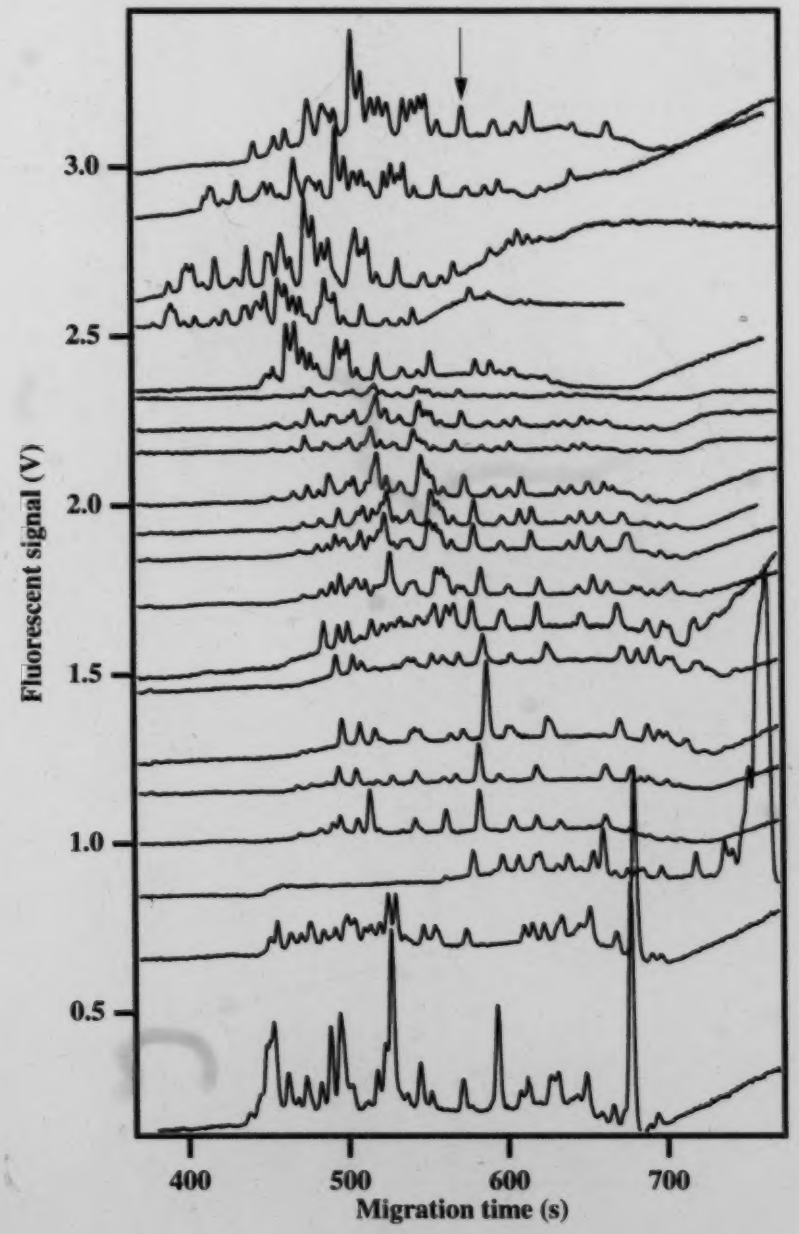


Figure 4.7 Time-adjusted electropherograms for the 20 runs using immobilized calf intestinal alkaline phosphatase on capillary no. CI-13. The runs are centered on the peak denoted with the arrow (central peak, CP), starting with run 1 at the bottom; run 8 is omitted.

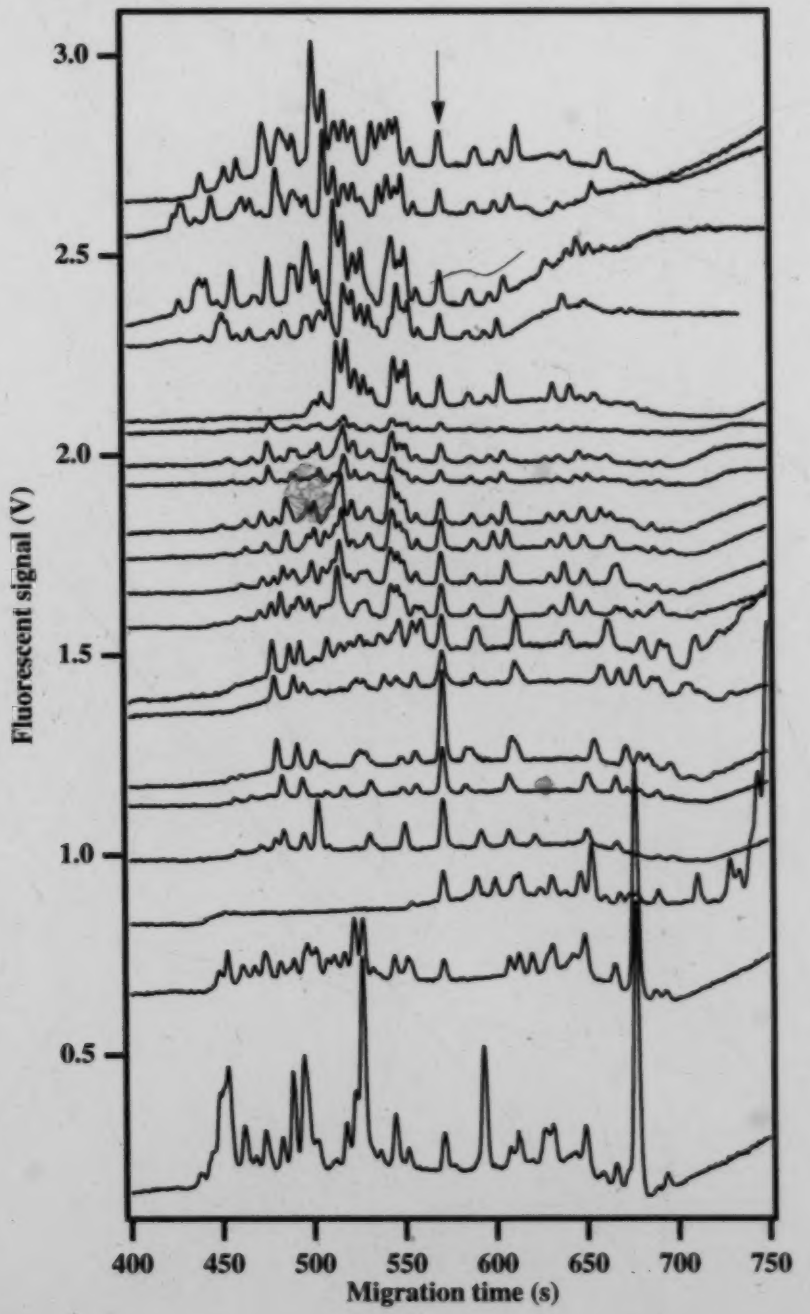


Table 4.3 Results of 20 runs performed on the immobilized molecules of calf intestinal alkaline phosphatase using capillary no. CI-13. Only the central peak (CP) persisted throughout all the runs. Peak descriptions: CP = central peak, P1-P6 = peaks 1-6.

		Peak description						
Run	Date	CP	P1	P2	P3	P4	P5	P6
1	05/06/2000	✓						
2	05/06/2000	✓						
3	05/06/2000	✓	✓	✓	✓			
4	06/06/2000	✓	✓	✓	✓			
5	06/06/2000	✓	✓		✓			
6	06/06/2000	✓						
7	06/06/2000	✓	✓			✓	✓	✓
9	06/06/2000	✓	✓		✓	✓	✓	✓
10	07/06/2000	✓	✓		✓	✓	✓	✓
11	07/06/2000	✓	✓		✓	✓	✓	✓
12	07/06/2000	✓	✓	✓	✓	✓	✓	✓
13	07/06/2000	✓	✓	✓	✓	✓	✓	✓
14	07/06/2000	✓	✓	✓	✓	✓	✓	✓
15	07/06/2000	✓	✓	✓	✓	✓	✓	✓
16	07/06/2000	✓	✓	✓	✓	✓	✓	✓
17	08/06/2000	✓	✓	✓	✓	✓	✓	✓
18	08/06/2000	✓	✓	✓	✓			
19	08/06/2000	✓	✓	✓	✓			
20	08/06/2000	✓	✓	✓	✓			
21	08/06/2000	✓	✓	✓	✓			

The peak areas in the electropherograms were measured using PeakFit. The data from each day was then fitted to a standard curve for that day to obtain the Fluorescent Emitter concentration. The standard curves were forced through the zero intercept, based on the assumption that the area above the baseline of a peak produced by an enzyme molecule in absence of substrate is zero. The standards for days 2-4 are shown in **Figure 4.8** along with slope and R^2 value information. The concentration of product in a given peak was then divided by the time of incubation to obtain the reaction velocity in M/s for the particular molecule. To calculate the reaction velocity in units of μmol/min., the following calculation was performed:

$$V_{rxn}\left[\frac{\mu mol}{\min}\right] = V_{rxn}\left[\frac{M}{s}\right] \times \frac{Vol_{inj}[L] \times 60\left[\frac{s}{\min}\right]}{1\times10^{-6}\left[\frac{mol}{\mu mol}\right]} \qquad \textbf{(Equation 4.1)}$$

where V_{rxn} is the reaction velocity and Vol_{inj} is the volume of injected standard peak. For capillary no. CI-13 this volume was measured to be 159 ± 8 pL.

The reaction velocities calculated for a molecule were then plotted against the concentration of the substrate and the curve was fitted in IgorPro (WaveMetrics,) using the Michaelis-Menten equation:

$$V_o = \frac{V_{max} \times [S]}{K_m + [S]} \qquad \textbf{(Equation 4.2)}$$

where V_o is the reaction velocity, V_{max} is the maximum reaction velocity, [S] is the substrate concentration and K_m is the Michaelis constant. The plots for the seven measured molecules, as well as for the average velocity of these seven molecules, are shown in **Figures 4.9** to **4.16**. The V_{max} and K_m values are shown in the graphs and also compiled in **Table 4.4**. In each case, **Figure a)** shows the data for the given molecule obtained over 3 days (days 2, 3 and 4) while **Figure b)** shows the data for the same molecule obtained in the last two days only. This distinction was made after observing that the second day contained some points which were clearly far removed from the fit.

Figure 4.8 Fits to standard data used in the calculation of the activities of alkaline phosphatase molecules form capillary no. CI-13.

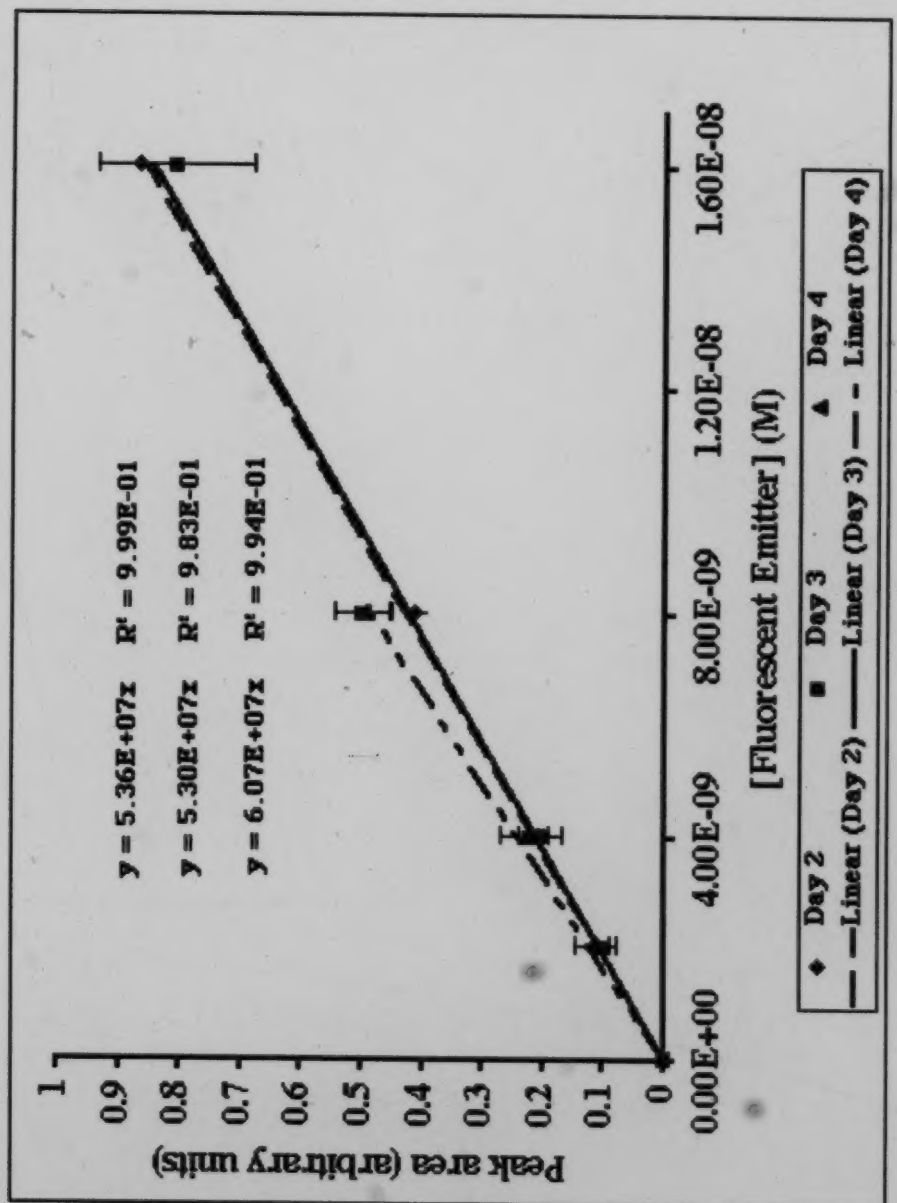
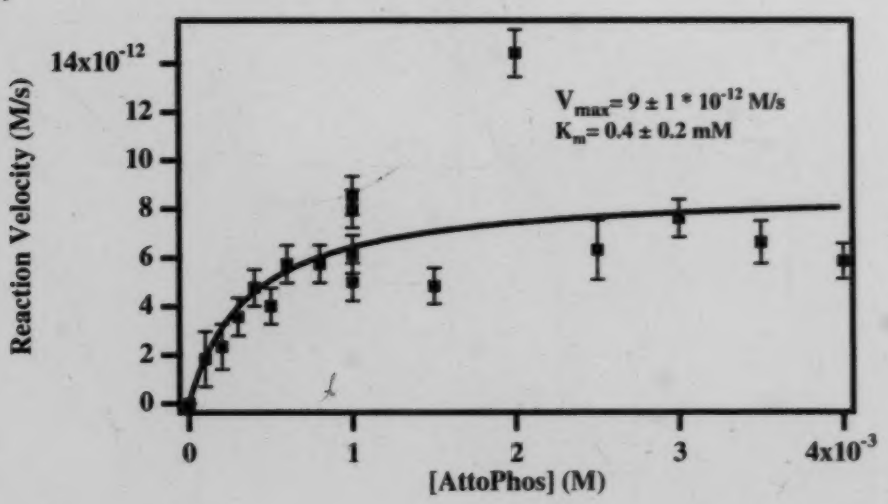


Figure 4.9 Michaelis-Menten plots of the activity of the CP molecule of alkaline phosphatase from capillary no. CI-13: **a)** data obtained from days 2-4, **b)** data obtained from days 3 and 4. Y-axis scaling varies.

a)



b)

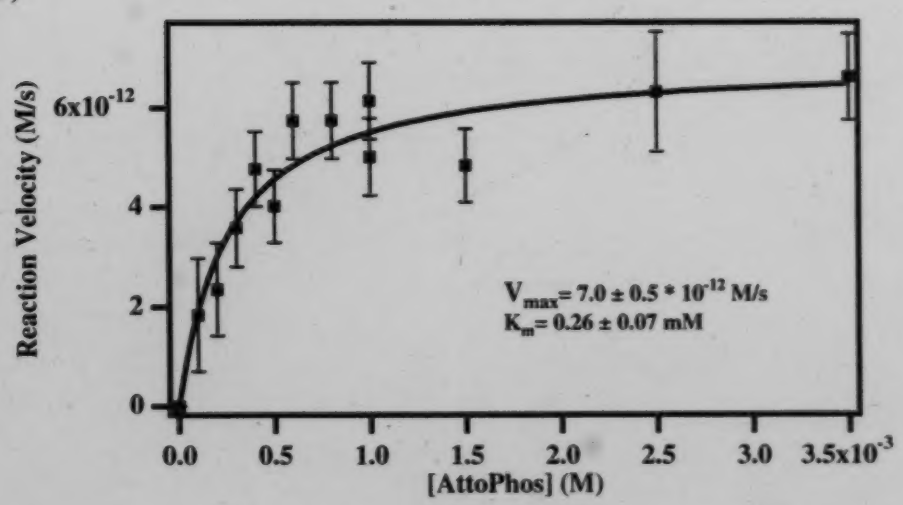


Figure 4.10 Michaelis-Menten plots of the activity of the P1 molecule of alkaline phosphatase from capillary no. CI-13: **a)** data obtained from days 2-4, **b)** data obtained from days 3 and 4. Y-axis scaling varies.

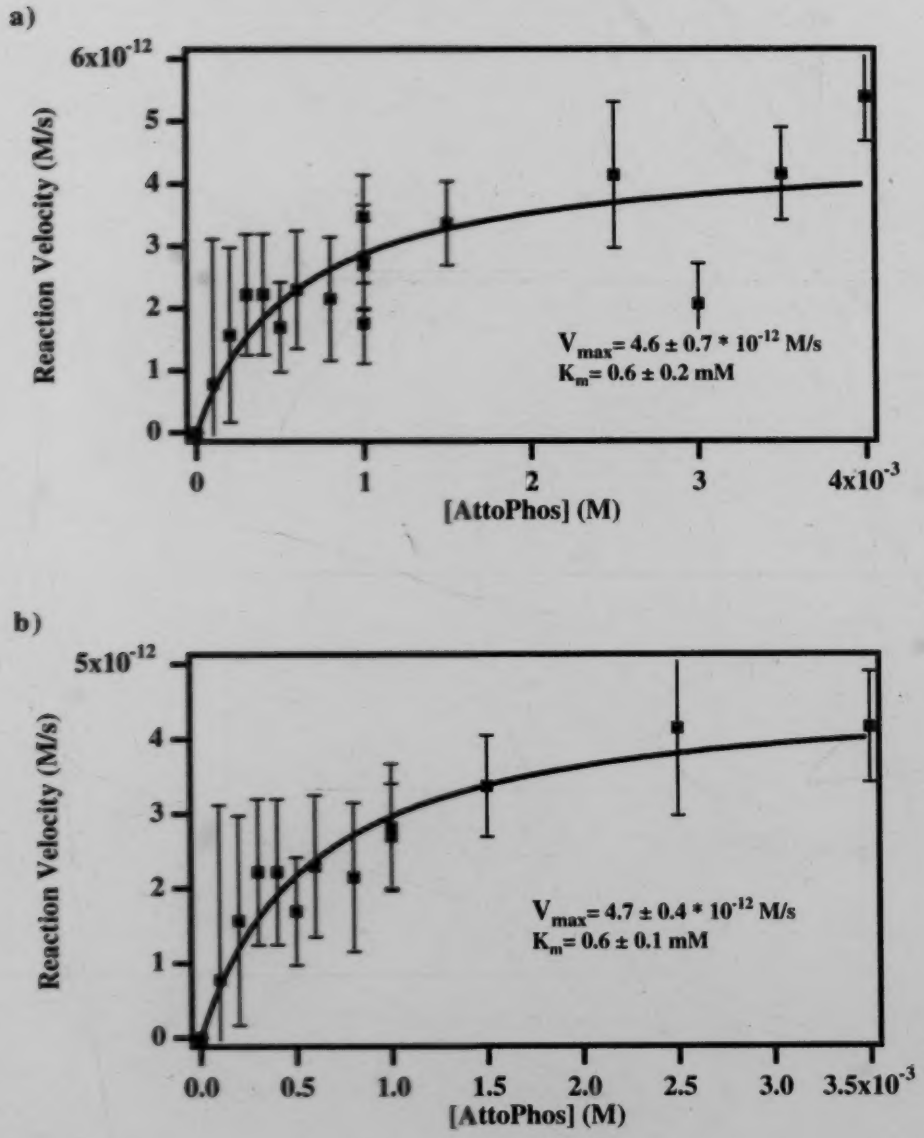


Figure 4.11 Michaelis-Menten plots of the activity of the P2 molecule of alkaline phosphatase from capillary no. CI-13: **a)** data obtained from days 2-4, **b)** data obtained from days 3 and 4. Y-axis scaling varies.

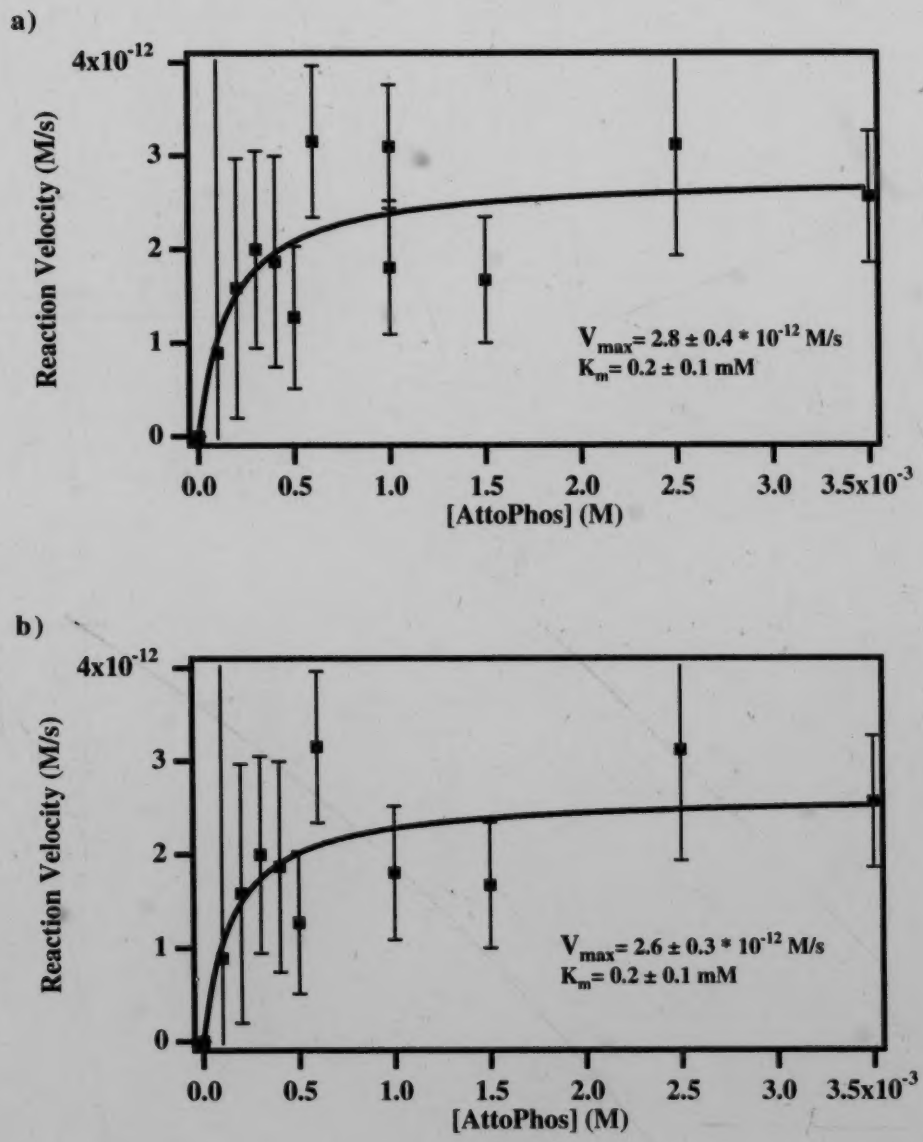


Figure 4.12 Michaelis-Menten plots of the activity of the P3 molecule of alkaline phosphatase from capillary no. CI-13: **a)** data obtained from days 2-4, **b)** data obtained from days 3 and 4. Y-axis scaling varies.

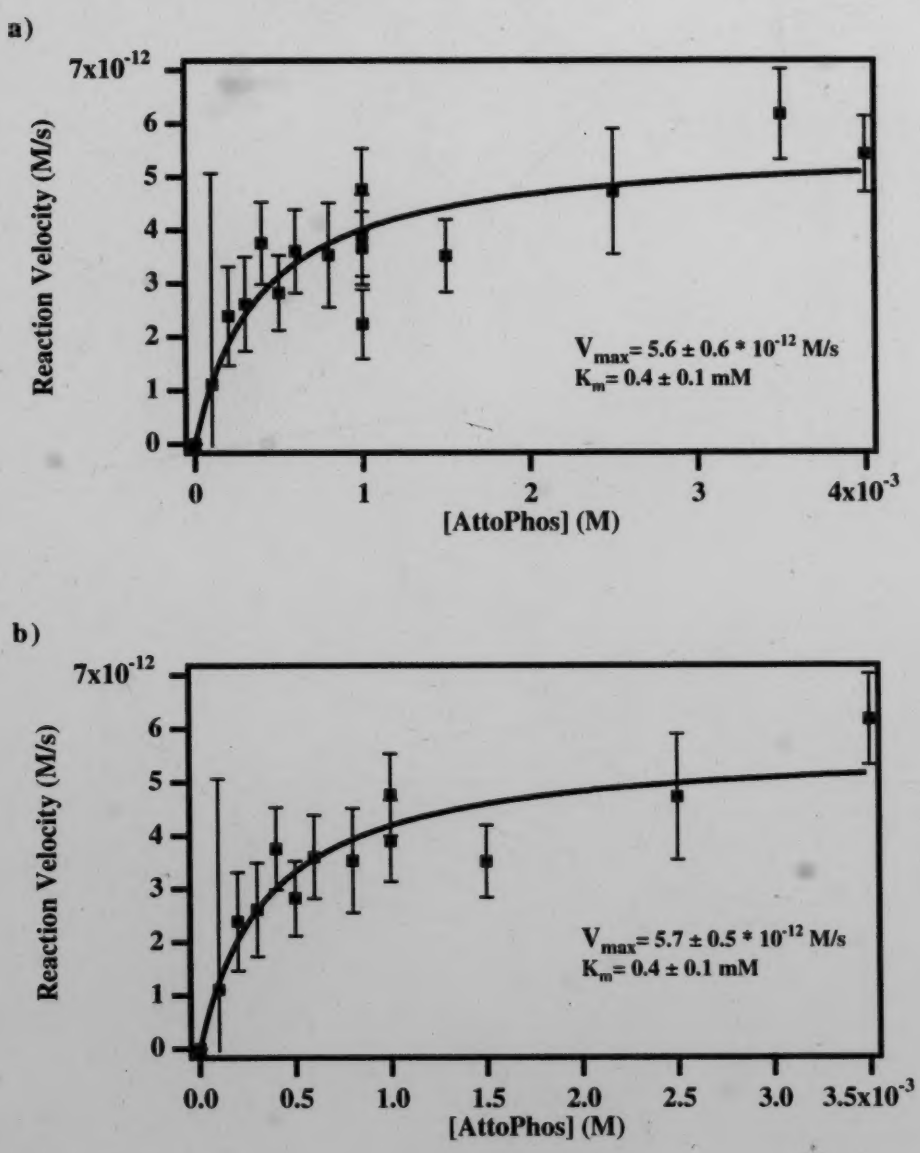
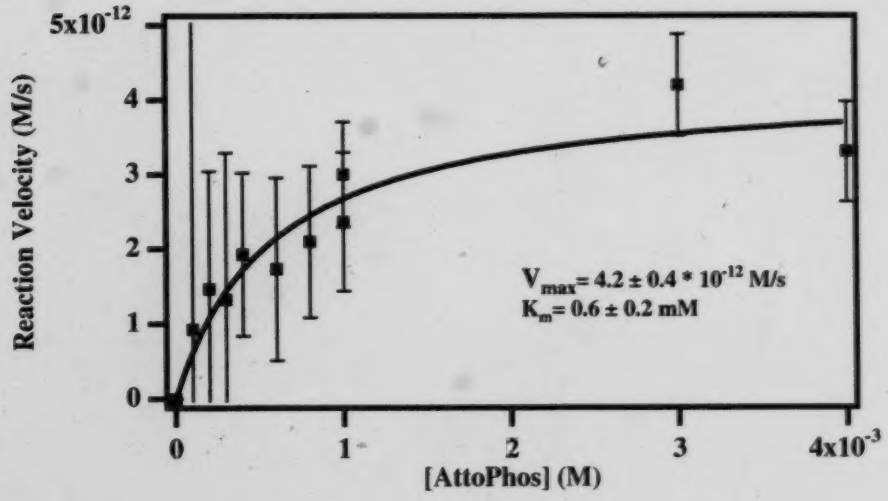


Figure 4.13 Michaelis-Menten plots of the activity of the P4 molecule of alkaline phosphatase from capillary no. CI-13: **a)** data obtained from days 2-4, **b)** data obtained from days 3 and 4. Y-axis scaling varies.

a)



b)

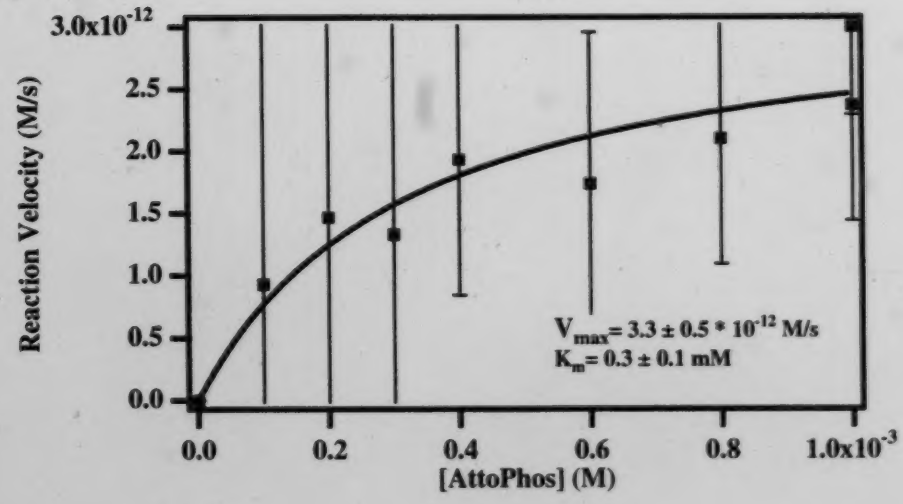


Figure 4.14 Michaelis-Menten plots of the activity of the P5 molecule of alkaline phosphatase from capillary no. CI-13: **a)** data obtained from days 2-4, **b)** data obtained from days 3 and 4. Y-axis scaling varies.

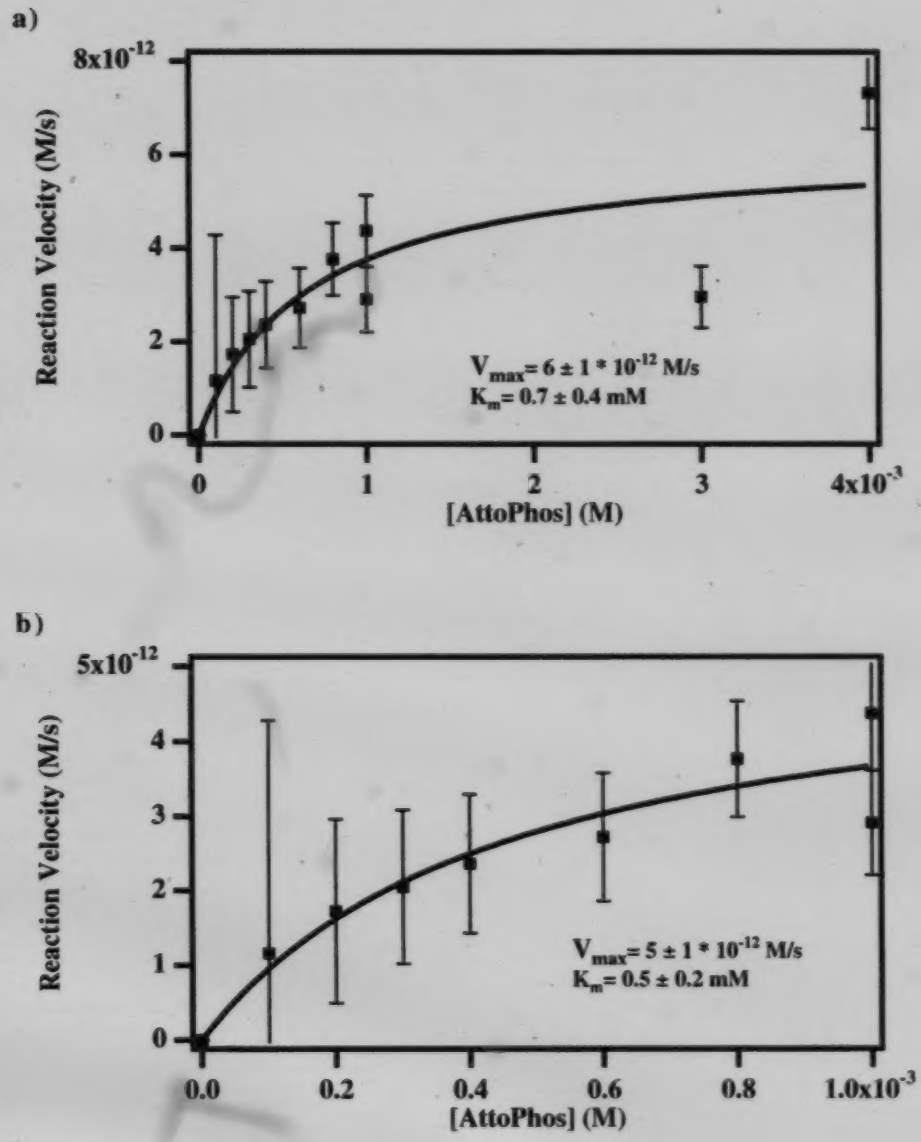
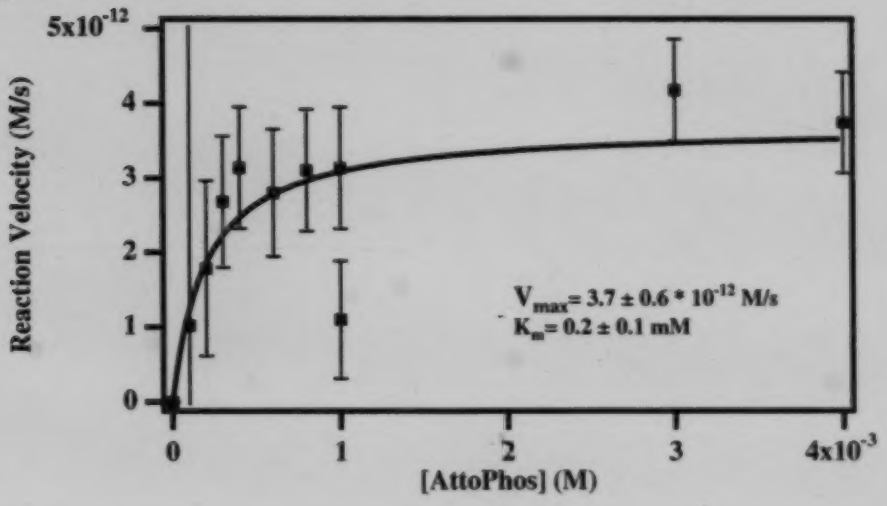


Figure 4.15 Michaelis-Menten plots of the activity of the P6 molecule of alkaline phosphatase from capillary no. CI-13: **a)** data obtained from days 2-4, **b)** data obtained from days 3 and 4. Y-axis scaling varies.

a)



b)

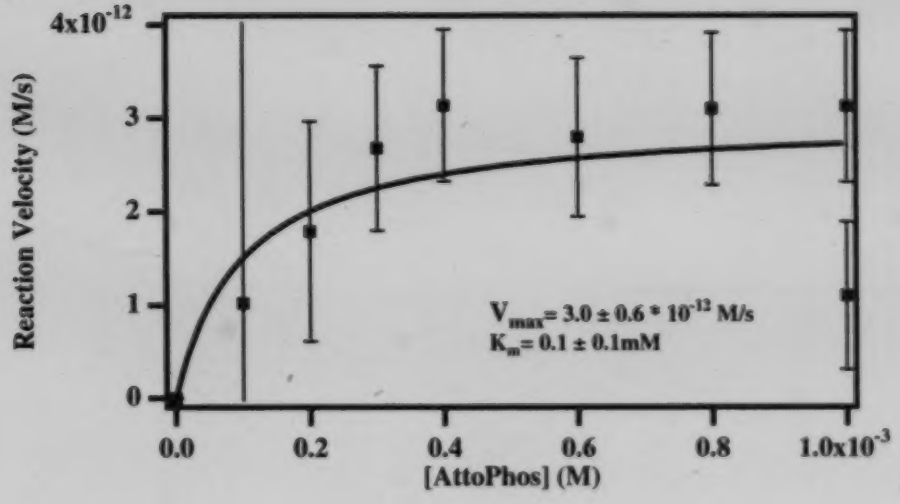
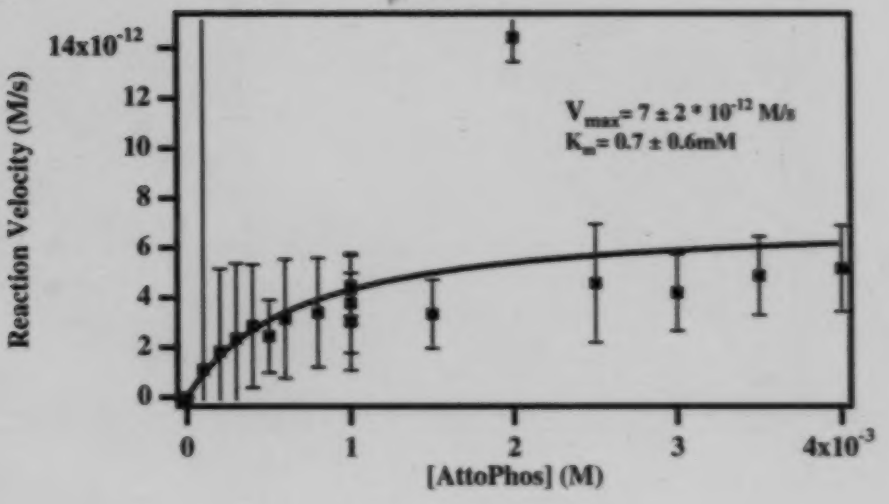


Figure 4.16 Michaelis-Menten plots of the average activity of all the studied molecules of alkaline phosphatase from capillary no. CI-13: **a)** data obtained from days 2-4, **b)** data obtained from days 3 and 4. Y-axis scaling varies.

a)



b)

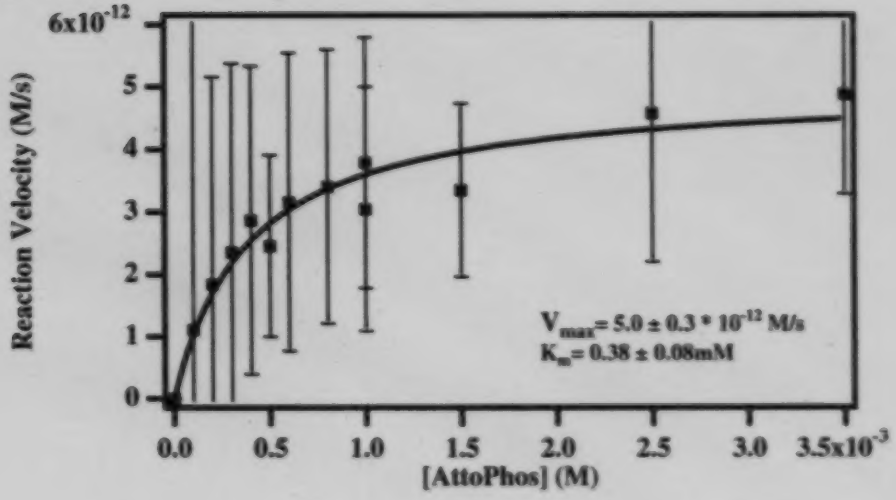


Table 4.4 **a)** Maximum reaction velocities and **b)** Michaelis constant values for molecules immobilized on capillary no. CI-13. All values are presented with one figure beyond significant, shown in offset case.

a)

Molecule	V_{max}			
	$\times 10^{-12}$ (M/s)		$\times 10^{-14}$ (µmol/min.)	
	Day 2-4	Day 3, 4	Day 2-4	Day 3, 4
CP	8.8 ± 1.4	$6.9_7 \pm 0.4_8$	8.4 ± 1.3	$6.6_5 \pm 0.4_6$
P1	$4.5_8 \pm 0.6_5$	$4.6_8 \pm 0.4_2$	$4.3_7 \pm 0.6_2$	$4.4_6 \pm 0.4_0$
P2	$2.7_8 \pm 0.4_0$	$2.6_3 \pm 0.3_9$	$2.6_5 \pm 0.3_8$	$2.5_1 \pm 0.3_7$
P3	$5.6_0 \pm 0.5_8$	$5.6_8 \pm 0.5_2$	$5.3_4 \pm 0.5_5$	$5.4_2 \pm 0.5_0$
P4	$4.2_3 \pm 0.4_4$	$3.2_6 \pm 0.5_3$	$4.0_4 \pm 0.4_2$	$3.1_1 \pm 0.5_1$
P5	6.2 ± 1.3	5.3 ± 1.0	5.9 ± 1.2	$5.0_6 \pm 0.9_5$
P6	$3.6_9 \pm 0.5_5$	$3.0_0 \pm 0.6_5$	$3.5_2 \pm 0.5_2$	$2.8_6 \pm 0.6_2$
P Average	7.2 ± 2.2	$4.9_8 \pm 0.3_1$	6.9 ± 2.1	$4.7_5 \pm 0.3_0$

b)

Molecule	K_m (mM)	
	Day 2-4	Day 3, 4
CP	$0.3_6 \pm 0.2_2$	$0.26_5 \pm 0.06_7$
P1	$0.6_0 \pm 0.2_5$	$0.5_8 \pm 0.1_4$
P2	$0.1_7 \pm 0.1_1$	$0.1_5 \pm 0.1_0$
P3	$0.4_0 \pm 0.1_4$	$0.3_6 \pm 0.1_1$
P4	$0.5_8 \pm 0.1_7$	$0.3_2 \pm 0.1_4$
P5	$0.6_6 \pm 0.3_6$	$0.4_6 \pm 0.2_1$
P6	$0.2_0 \pm 0.1_2$	$0.1_0 \pm 0.1_1$
P Average	$0.6_7 \pm 0.6_0$	$0.38_5 \pm 0.07_7$

The second study of the kinetics of calf intestinal alkaline phosphatase was a more thorough follow-up to the previous study. The electropherograms obtained in these assays are shown in **Figures 4.17** to **4.22**. Capillary no. CI-18 contained 6 alkaline phosphatase molecules, although rather poorly resolved. The number of peaks was estimated by minimizing residuals while fitting between 3 and 6 peaks to the curve. Minimal residual values were obtained with 6 peaks fitted. An additional peak appeared in the first four runs of the second day of capillary's use. This peak later disappeared again, leading to a conclusion that it must have been a contaminating molecule of alkaline phosphatase. The capillary was used despite the poor resolution because the molecules proved to be very resilient, lasting for 63 runs over 7 days. Data for the first and last day were not included in the analysis: there were no standards for the first day and only four runs were done on the last day, none of them in replicate.

The data obtained with this capillary was analyzed similarly to the data obtained with the previous capillary (see above). The standards for the five days of analysis are shown in **Figure 4.23** along with slope and R^2 values. To convert the reaction velocity from units of M/s to μmol/min., **Equation 4.1** was used with Vol_{inj} of 198 ± 10 pL. The calculated velocities were plotted against substrate concentration and fitted with **Equation 4.2** using IgorPro. **Figures 4.24** to **4.30** show the Michaelis-Menten plots for the 6 analyzed molecules and for the average activity of all the molecules. In each case, **Fig. a)** shows all the data points obtained for a given molecule over the five days, while **Fig. b)** shows the average value for each substrate concentration. The error bars correspond to error associated with peak measurements and to standard deviation in the average value, respectively. Each plot also shows two fits - one for the data obtained over 5 days (days 2-6) and the other for the data which excludes day 3. **Table 4.5** shows the results of the above analysis of data from this capillary.

Figure 4.17 Time adjusted electropherograms obtained from capillary no. CI-18. The capillary contained 6 immobilized molecules of calf intestinal alkaline phosphatase. The electropherograms are centered on molecule no. 5, the most active one.

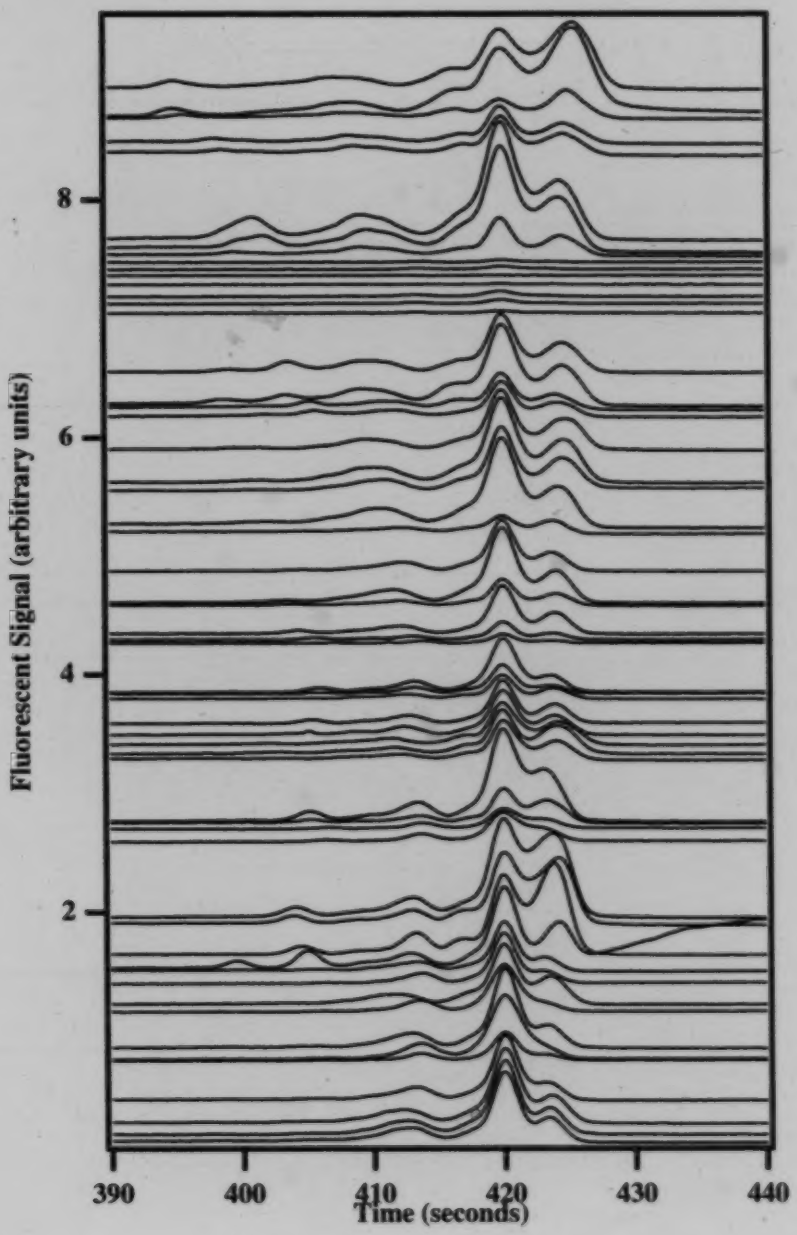


Figure 4.18 Electropherograms of runs obtained using capillary no. CI-18 and [AttoPhos™] of 1 μM to 50 μM. Starting from the bottom trace, the [AttoPhos™] and incubation times are: (1.0 μM, 1200 s);(5.0 μM: 600 s, 1200 s, 1800 s);(10.0 μM: 600 s, 1200 s, approximately 1800 s);(50.0 μM: 600 s, 1200 s, 1800 s).

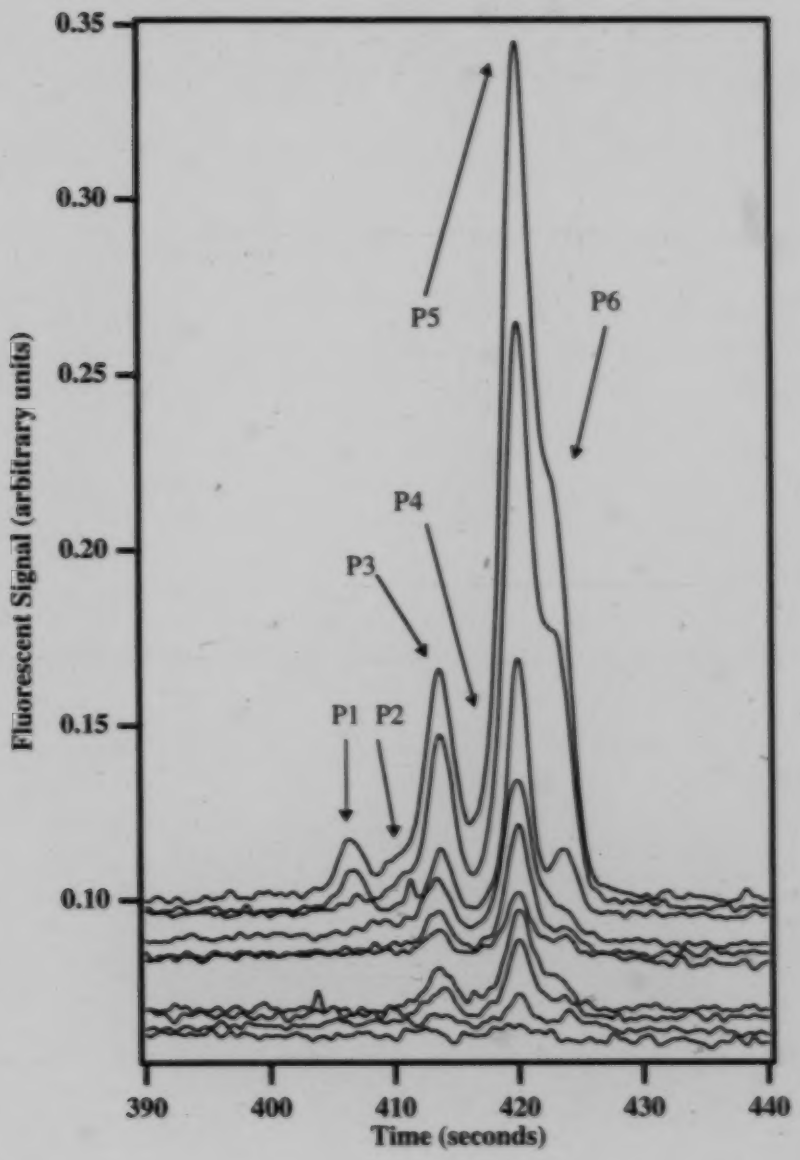


Figure 4.19 Electropherograms of runs obtained using capillary no. CI-18 and [AttoPhos™] of 0.1 mM to 0.9 mM. Starting from the bottom trace, the [AttoPhos™] and incubation times are: (0.10 mM: 930 s, 1800 s, 2700 s);(0.30 mM: 600 s, 900 s, 1230 s);(0.50 mM: 300 s, 610 s, 900 s);(0.70 mM: 600 s, 1200 s, 1800 s);(0.90 mM: 300 s, 600 s, 900s).

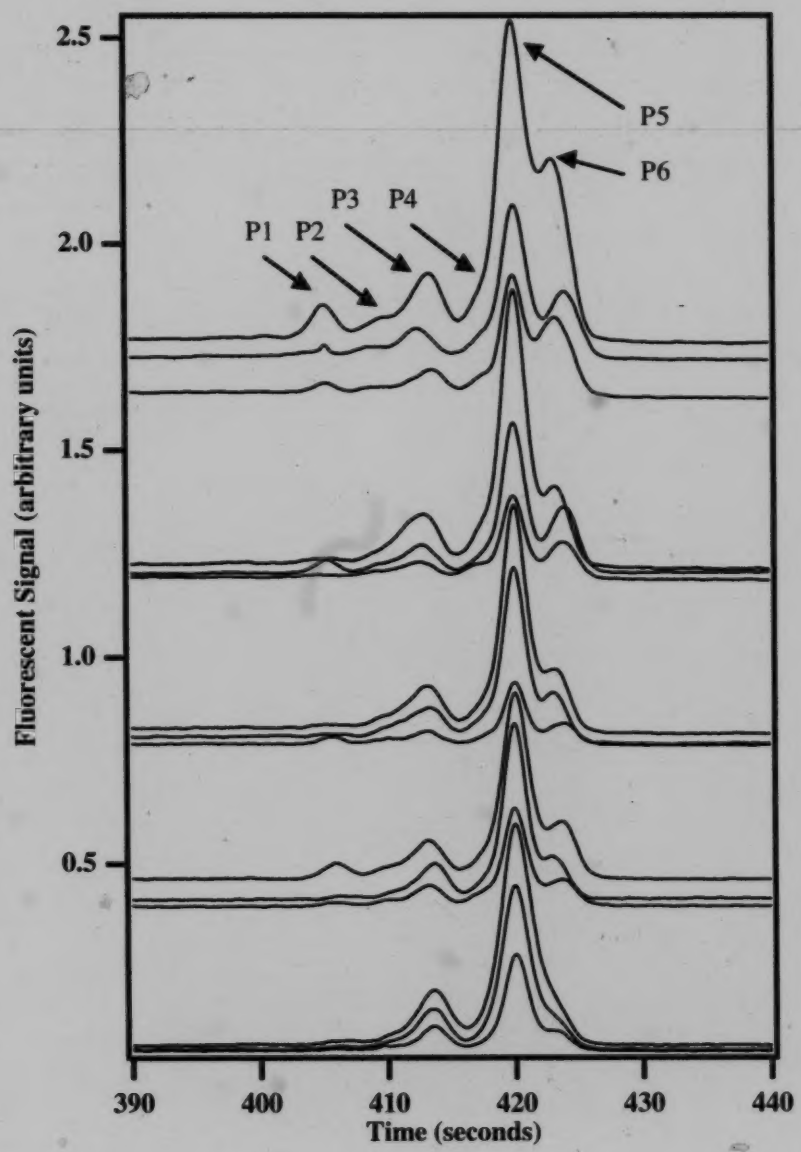


Figure 4.20 Electropherograms of runs obtained using capillary no. CI-18 and [AttoPhos™] of 1.0 mM to 1.7 mM. Starting from the bottom trace, the [AttoPhos™] and incubation times are: (1.0 mM: 630 s, 905 s, 920 s);(1.2 mM: 300 s, 600 s, 900 s);(1.5 mM: 300 s, 600 s, 910 s);(1.7 mM: 300 s, 600 s, 900 s).

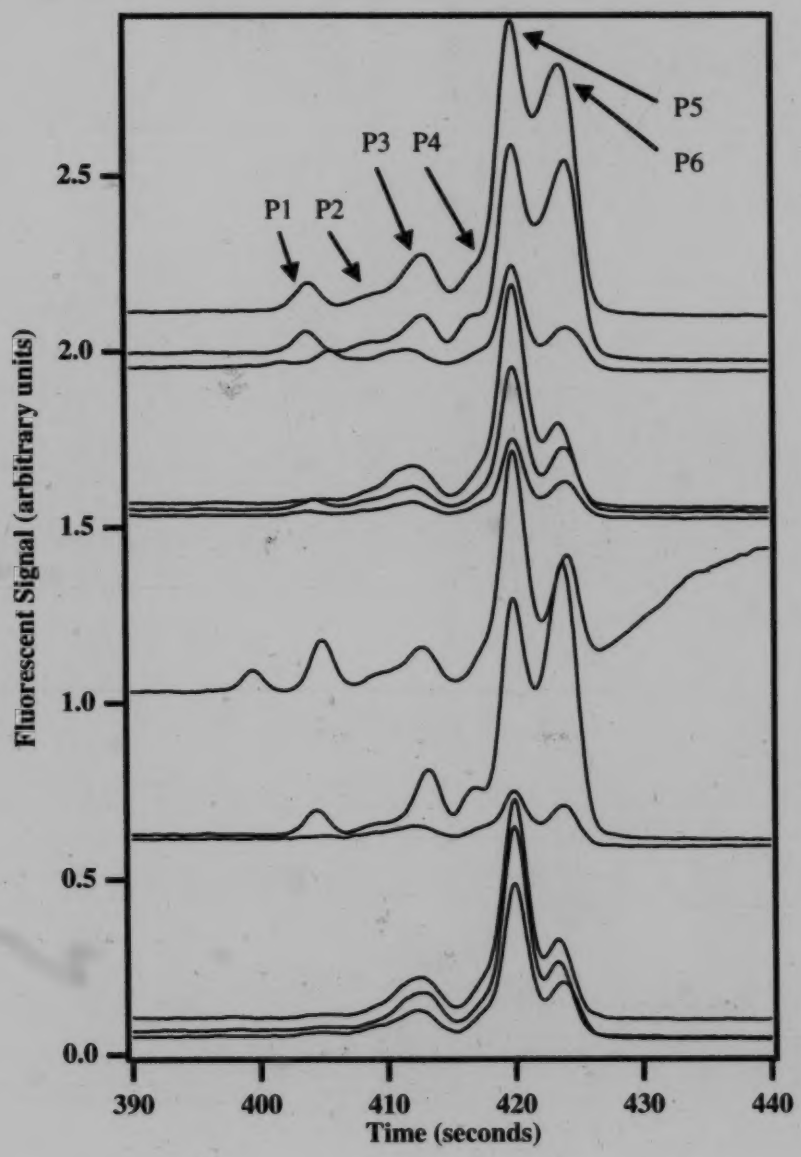


Figure 4.21 Electropherograms of runs obtained using capillary no. CI-18 and [AttoPhos™] of 2.0 mM to 3.5 mM. Starting from the bottom trace, the [AttoPhos™] and incubation times are: (2.0 mM: 300 s, 600 s, 900 s);(2.5 mM: 300 s, 620 s, 900 s);(3.0 mM: 300 s, 600 s, 900 s);(3.5 mM: 330 s, 600 s, 900 s).

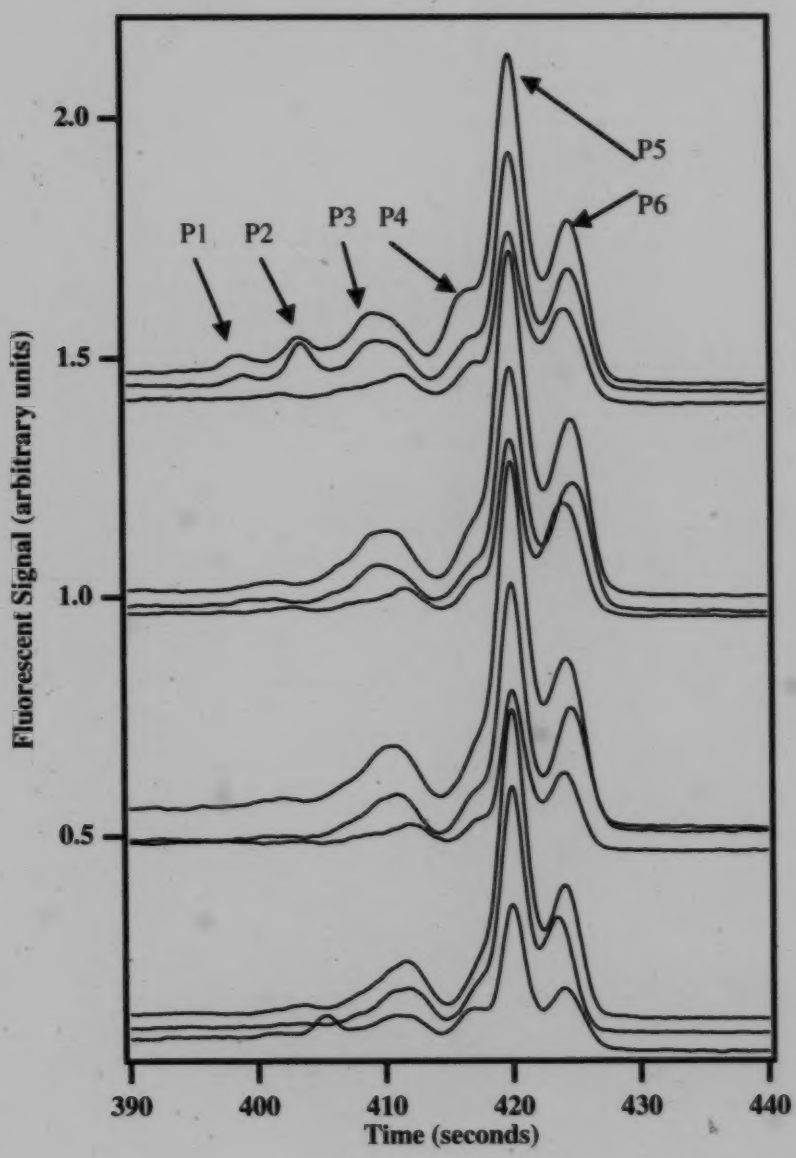


Figure 4.22 Electropherograms of runs obtained using capillary no. CI-18 and [AttoPhos™] of 4.0 mM to 7.0 mM. Starting from the bottom trace, the [AttoPhos™] and incubation times are: (4.0 mM: 300 s, 960 s);(4.5 mM: 1200 s);(5.0 mM: 310 s);(5.5 mM: 300 s);(6.0 mM: 305 s);(6.5 mM, 900 s);(7.0 mM, 900 s).

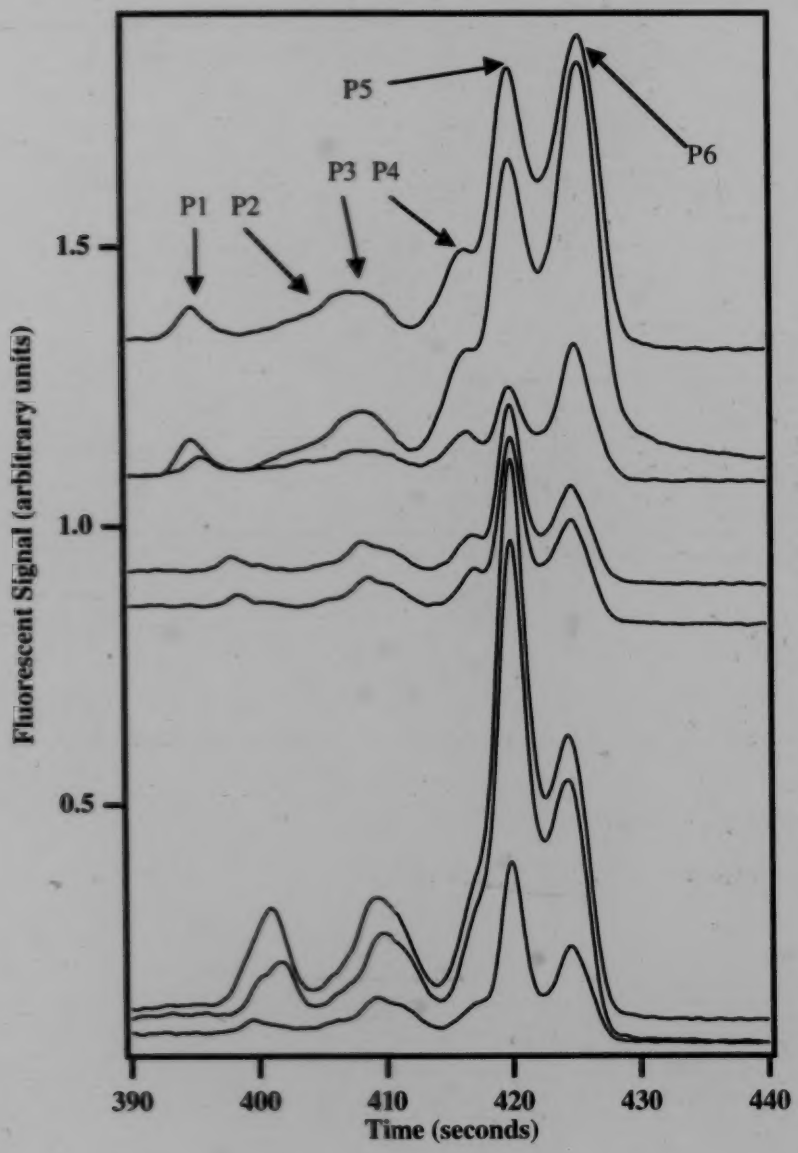


Figure 4.23 Fits to standard data used in the calculation of the activities of alkaline phosphatase molecules form capillary no. CI-18.

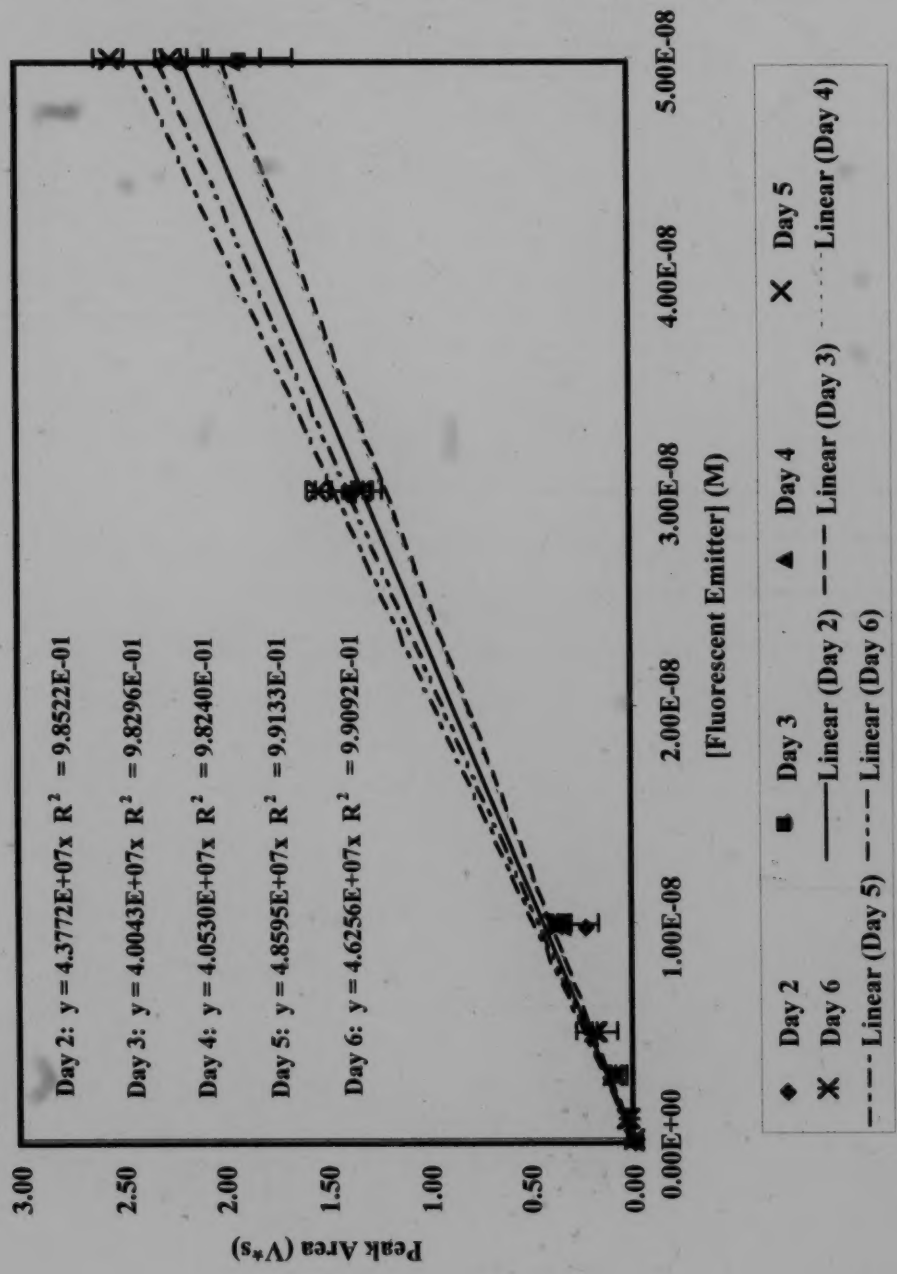


Figure 4.24 Michaelis-Menten plot for molecule P1 immobilized on capillary CI-18 (the solid line is the fit to all data; the broken line is the fit to data which excludes day 3): **a)** scatter plot of all the data points (error bars correspond to errors in velocity measurements), **b)** plot of average reaction velocities (error bars correspond to standard deviation).

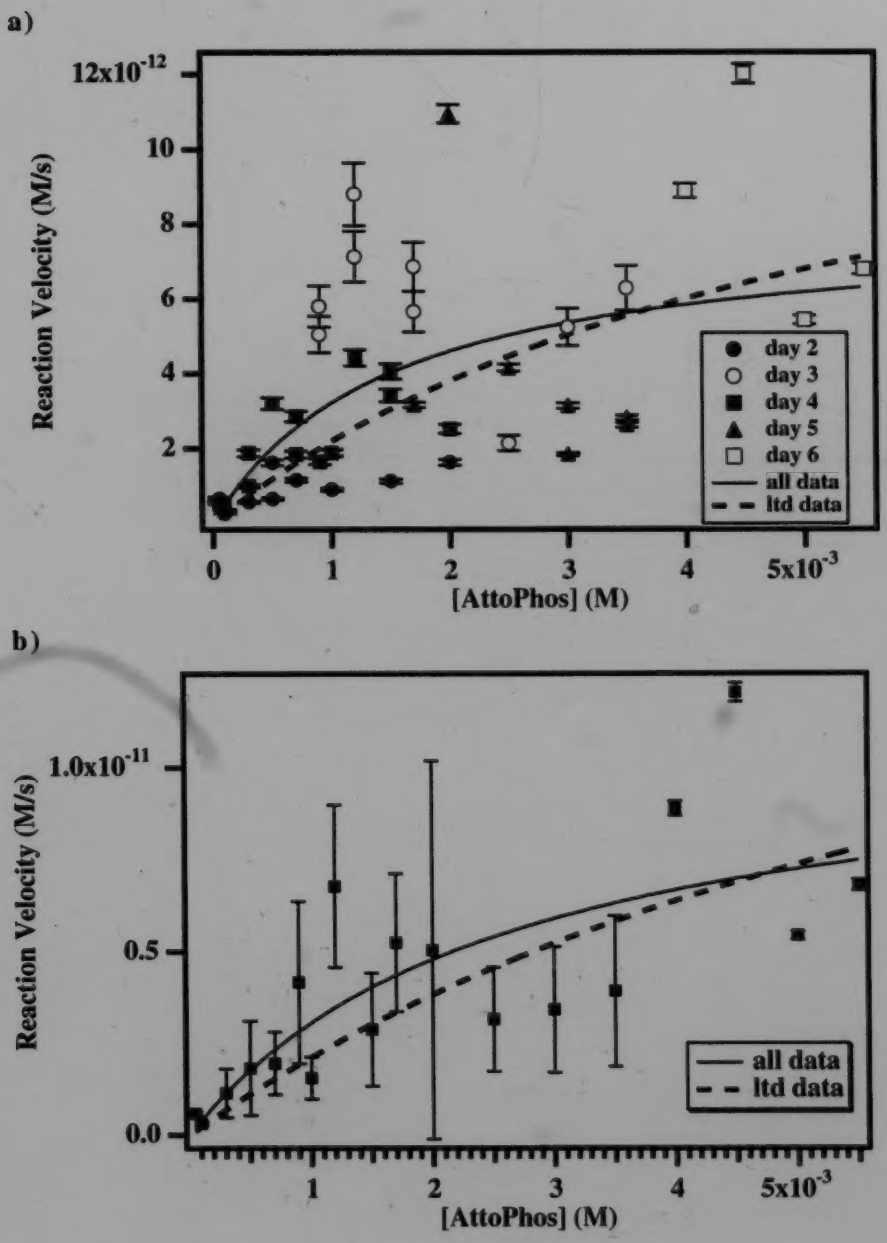
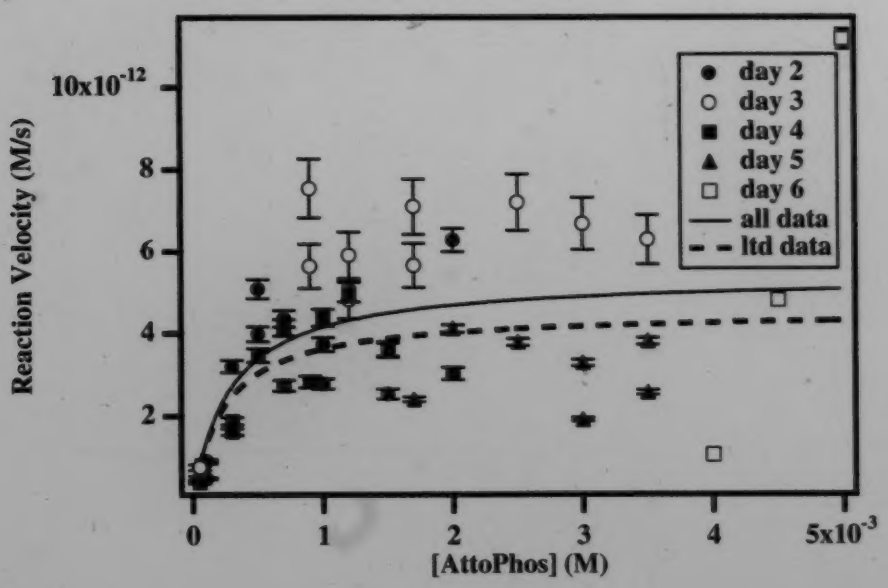


Figure 4.25 Michaelis-Menten plot for molecule P2 immobilized on capillary CI-18 (the solid line is the fit to all data; the broken line is the fit to data which excludes day 3): **a)** scatter plot of all the data points (error bars correspond to errors in velocity measurements), **b)** plot of average reaction velocities (error bars correspond to standard deviation).

a)



b)

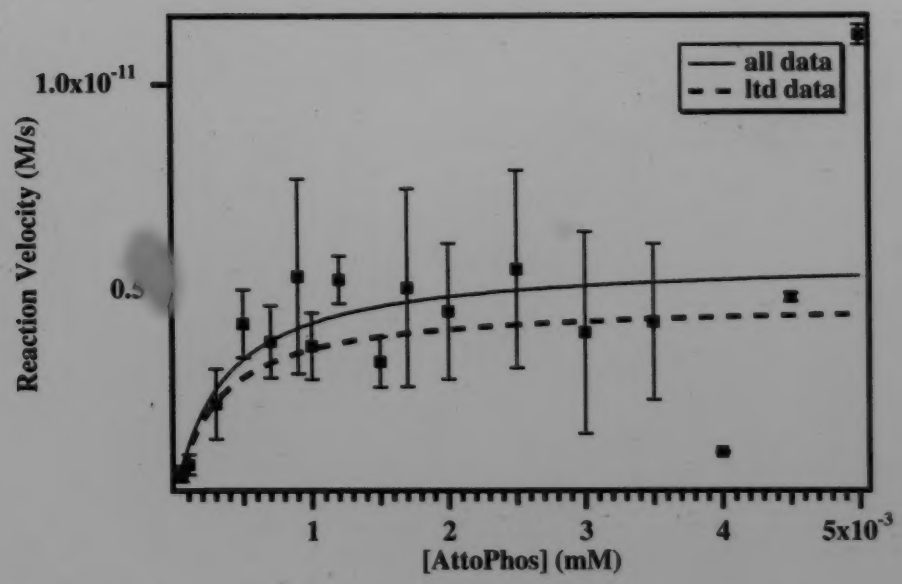


Figure 4.26 Michaelis-Menten plot for molecule P3 immobilized on capillary CI-18 (the solid line is the fit to all data; the broken line is the fit to data which excludes day 3): **a)** scatter plot of all the data points (error bars correspond to errors in velocity measurements), **b)** plot of average reaction velocities (error bars correspond to standard deviation).

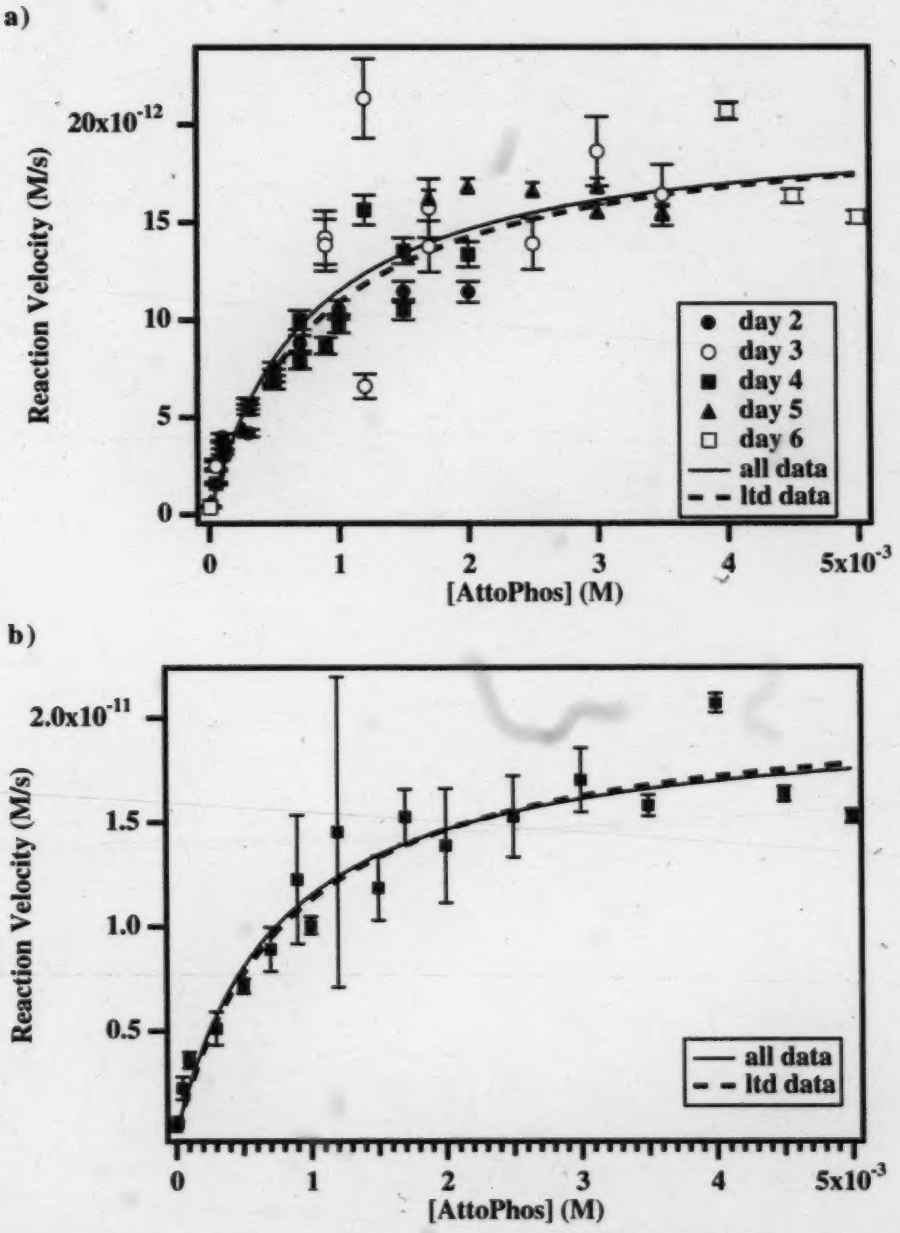
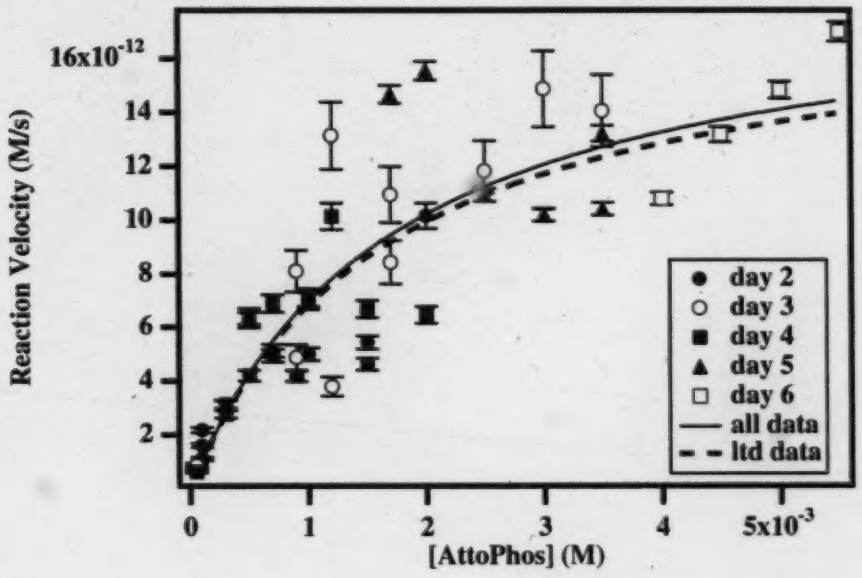


Figure 4.27 Michaelis-Menten plot for molecule P4 immobilized on capillary CI-18 (the solid line is the fit to all data; the broken line is the fit to data which excludes day 3): **a)** scatter plot of all the data points (error bars correspond to errors in velocity measurements), **b)** plot of average reaction velocities (error bars correspond to standard deviation).

a)



b)

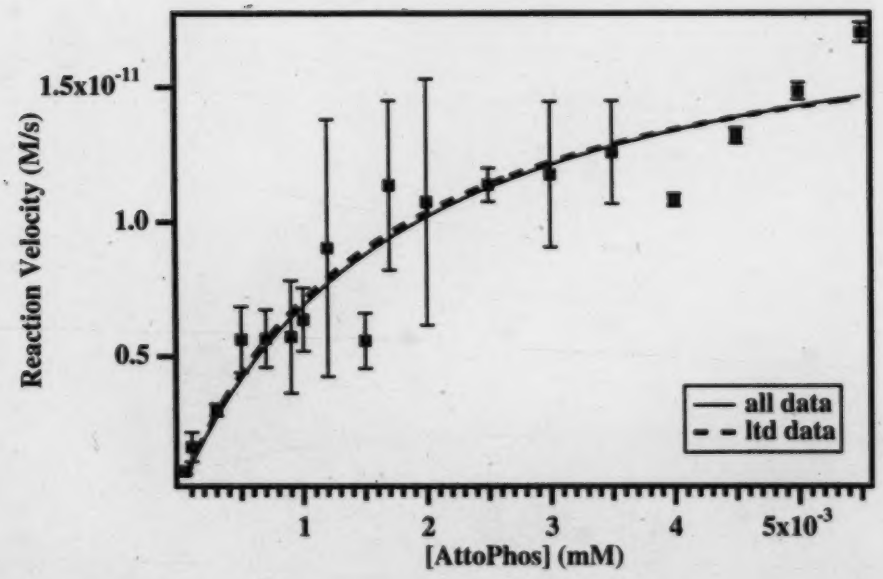
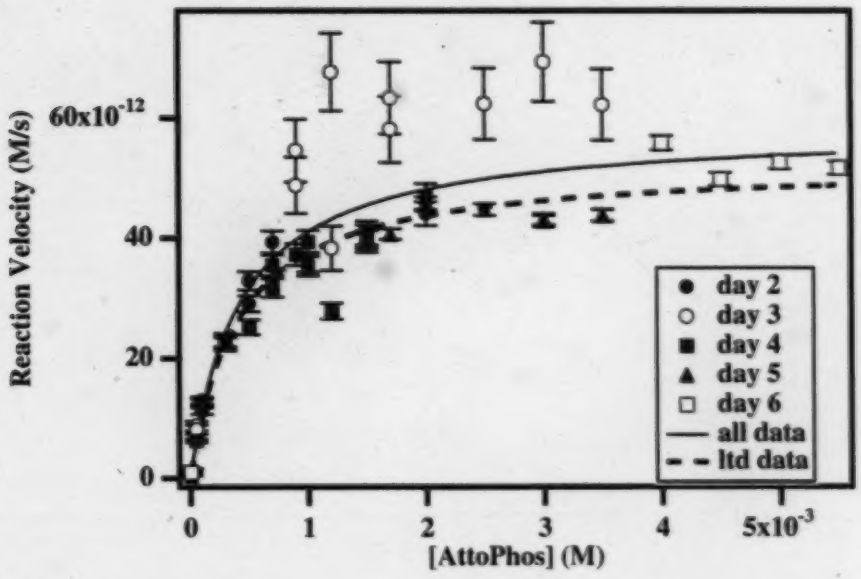


Figure 4.28 Michaelis-Menten plot for molecule P5 immobilized on capillary CI-18 (the solid line is the fit to all data; the broken line is the fit to data which excludes day 3): **a)** scatter plot of all the data points (error bars correspond to errors in velocity measurements), **b)** plot of average reaction velocities (error bars correspond to standard deviation).

a)



b)

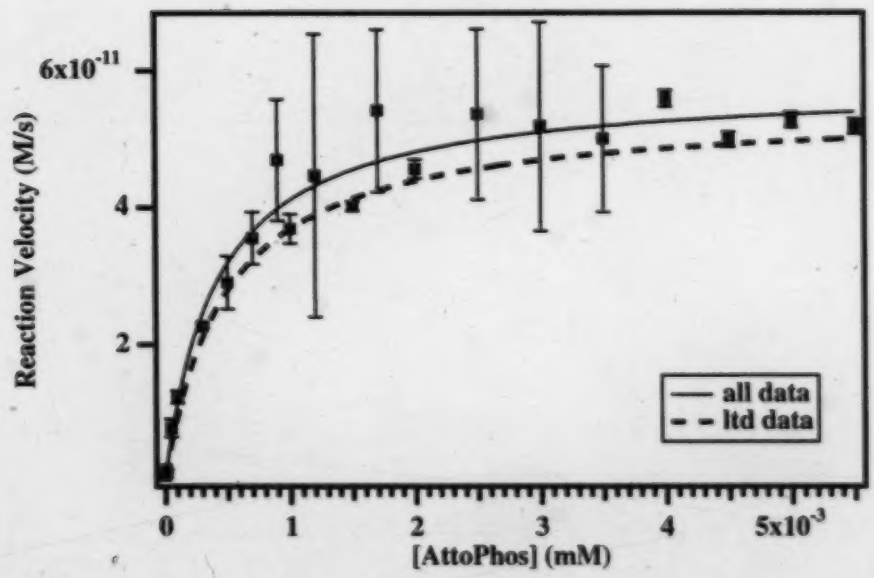


Figure 4.29 Michaelis-Menten plot for molecule P6 immobilized on capillary CI-18 (the solid line is the fit to all data; the broken line is the fit to data which excludes day 3): **a)** scatter plot of all the data points (error bars correspond to errors in velocity measurements), **b)** plot of average reaction velocities (error bars correspond to standard deviation).

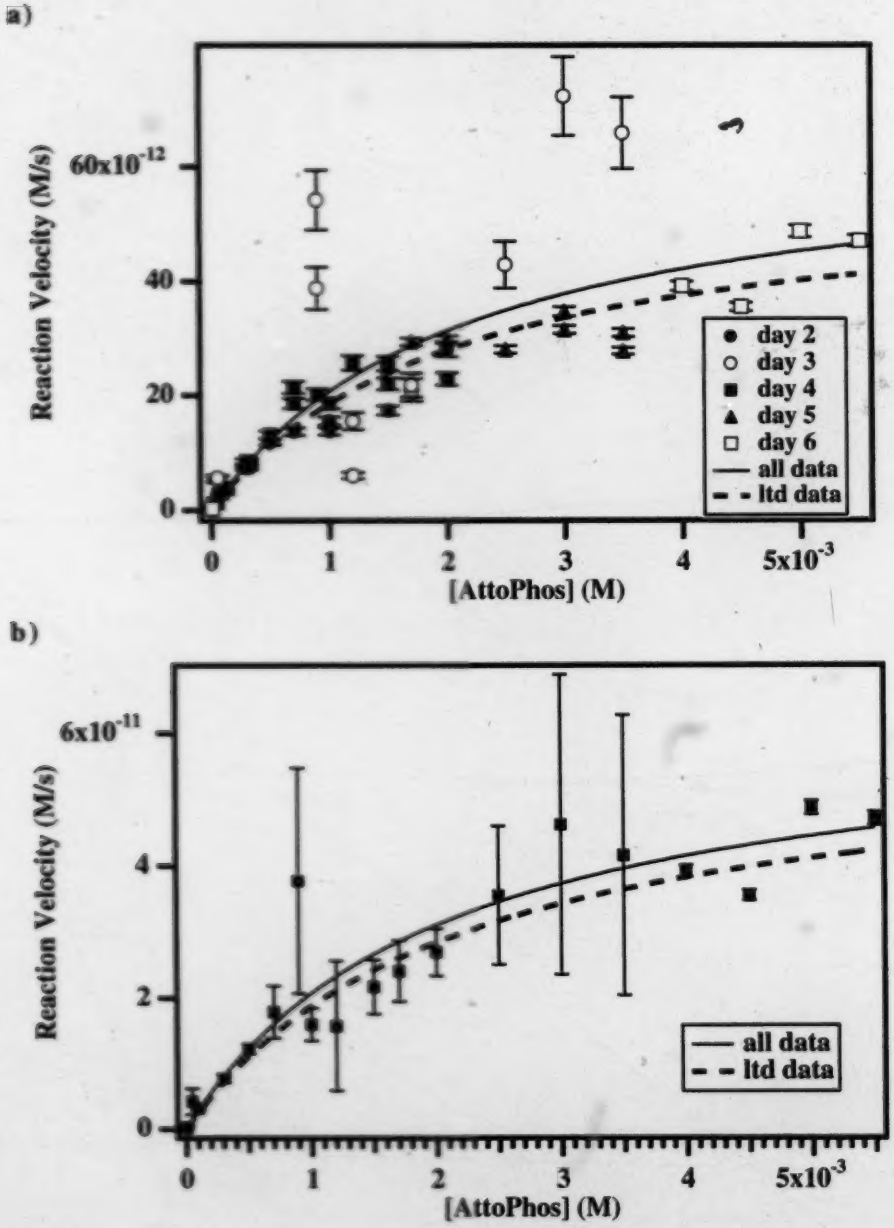
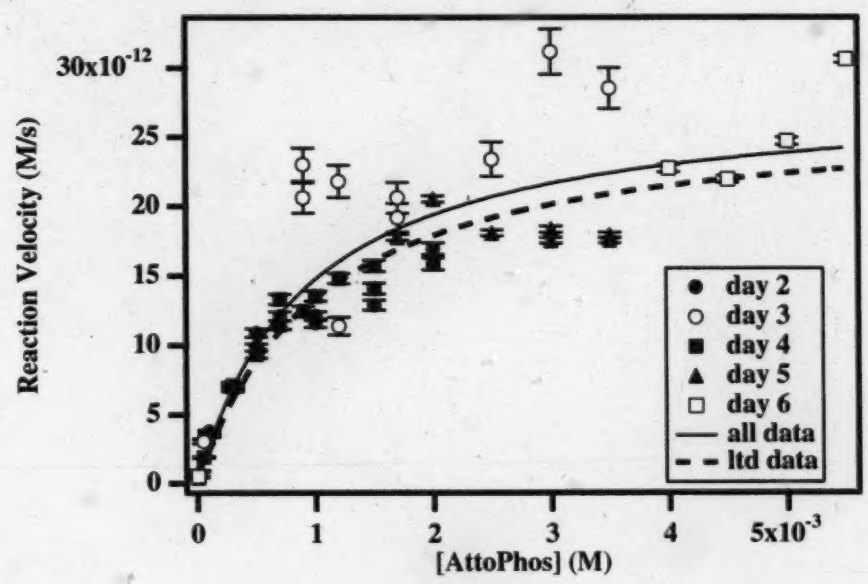


Figure 4.30 Michaelis-Menten plot for the average reaction velocity of all molecules immobilized on capillary CI-18 (the solid line is the fit to all data; the broken line is the fit to data which excludes day 3): **a)** scatter plot of all the data points (error bars correspond to errors in velocity measurements), **b)** plot of average reaction velocities (error bars correspond to standard deviation).

a)



b)

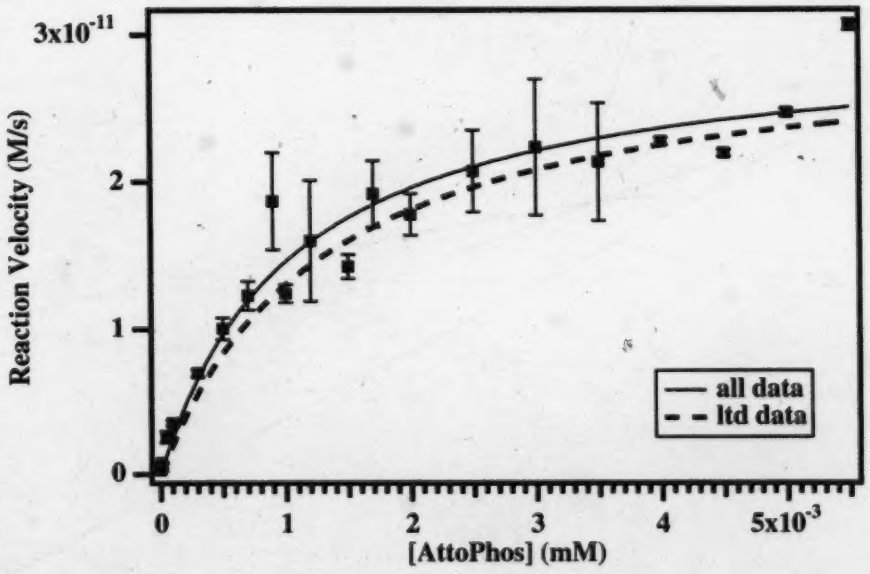


Table 4.5 Maximum reaction velocities for molecules immobilized on capillary no. CI-18: **a)** values obtained from scatter plots of all the data; **b)** values obtained from plots of average velocities; **c)** Michaelis constant values for the above molecules. All values are presented with one figure beyond significant, shown in offset case.

a)

Molecule	V_{max}			
	$\times 10^{-12}$ (M/s)		$\times 10^{-14}$ (µmol/min.)	
	Day 2-6	Day 2, 4-6	Day 2-6	Day 2, 4-6
P1	8.0 ± 2.1	14.3 ± 8.6	9.5 ± 2.5	17 ± 10
P2	5.38 ± 0.65	4.52 ± 0.64	6.39 ± 0.77	5.37 ± 0.76
P3	20.2 ± 1.4	20.5 ± 1.2	24.0 ± 1.7	24.4 ± 1.4
P4	18.9 ± 2.3	18.2 ± 2.4	22.4 ± 2.7	21.6 ± 2.8
P5	58.2 ± 3.1	52.4 ± 1.4	69.1 ± 3.7	62.3 ± 1.7
P6	64.4 ± 12.6	56.6 ± 4.6	76.5 ± 15.0	67.2 ± 5.5
P Average	28.2 ± 2.1	26.9 ± 1.5	33.5 ± 2.5	32.0 ± 1.8

b)

Molecule	V_{max}			
	$\times 10^{-12}$ (M/s)		$\times 10^{-14}$ (µmol/min.)	
	Day 2-6	Day 2, 4-6	Day 2-6	Day 2, 4-6
P1	11.0 ± 4.1	19 ± 17	13.1 ± 4.9	23 ± 20
P2	5.7 ± 1.2	4.7 ± 1.2	6.8 ± 1.4	5.6 ± 1.4
P3	20.2 ± 1.3	20.8 ± 1.6	24.0 ± 1.5	24.7 ± 1.9
P4	19.5 ± 2.0	19.0 ± 2.7	23.2 ± 2.4	22.6 ± 3.2
P5	57.8 ± 1.9	54.2 ± 2.2	68.7 ± 2.3	64.4 ± 2.6
P6	62.3 ± 9.4	58.6 ± 6.3	74.0 ± 11.2	69.6 ± 7.5
P Average	29.8 ± 2.0	29.7 ± 2.4	35.4 ± 2.4	35.3 ± 2.9

Table 4.5 Maximum reaction velocities for molecules immobilized on capillary no. CI-18: **a)** values obtained from scatter plots of all the data; **b)** values obtained from plots of average velocities; **c)** Michaelis constant values for the above molecules. All values are presented with one figure beyond significant, shown in offset case.

c)

	K_m (mM)			
Molecule	Scattered data		Averaged data	
	Day 2-6	Day 2, 4-6	Day 2-6	Day 2, 4-6
P1	$1.4_9 \pm 0.9_3$	$5._5 \pm 5._1$	$2._6 \pm 2._0$	$8._1 \pm 10._4$
P2	$0.2_9 \pm 0.1_7$	$0.2_5 \pm 0.1_7$	$0.3_6 \pm 0.3_5$	$0.3_1 \pm 0.4_0$
P3	$0.7_6 \pm 0.1_6$	$0.8_9 \pm 0.1_4$	$0.7_5 \pm 0.1_6$	$0.8_4 \pm 0.2_0$
P4	$1.7_1 \pm 0.4_7$	$1.6_7 \pm 0.5_0$	$1.8_4 \pm 0.4_6$	$1.6_8 \pm 0.6_1$
P5	$0.41_8 \pm .09_2$	$0.39_8 \pm 0.04_4$	$0.40_7 \pm .06_4$	$0.47_2 \pm 0.08_6$
P6	$2.1_2 \pm 0.8_5$	$2.0_6 \pm 0.3_6$	$2.0_2 \pm 0.7_1$	$2.1_3 \pm 0.5_2$
P Average	$0.9_2 \pm 0.2_0$	$1.0_1 \pm 0.1_6$	$1.0_5 \pm 0.2_2$	$1.2_8 \pm 0.2_9$

4.3.2.6 Results of the bulk kinetic assays on free calf intestinal alkaline phosphatase

The calibration curve for the bulk kinetic assay is shown in **Figure 4.31** along with the slope and R^2 values. The data obtained for the enzyme was fitted to this curve and divided by the time of incubation (15 min.) to obtain the activity of the enzyme in μmol/min. The Michaelis-Menten plot of the reaction velocities over a full substrate concentration range and fits to the data over eight consecutive concentration ranges are shown in **Figure 4.32**. The consecutive fits were necessary because of observed inhibition in alkaline phosphatase activity which becomes noticeable for [AttoPhos™] of between 1.0 and 1.7 mM. The values obtained for V_{max} (total and specific activity) and K_m over these ranges are shown in **Table 4.6**. The specific activity, per molecule of enzyme, is calculated using the enzyme concentration in the wells of $(1.39 \pm 0.04) \times 10^{-12}$ M, or $(4.9 \pm 0.2) \times 10^{-16}$ moles/well. Each well therefore contained $(2.9 \pm 0.1) \times 10^{8}$ molecules of alkaline phosphatase.

4.3.2.7 The effects of immobilization on the activity of calf intestinal alkaline phosphatase based on single molecule studies

The immobilization of active enzyme in 10 μm i.d. capillaries was generally successful. Although not every attempt ended with a capillary containing both active and useful molecules, it never took more than 2 or 3 attempts to produce a working reactor. The attempts to make the reactor well defined, i.e. to immobilize the molecules within a strictly defined window, were more problematic. This was probably due to two factors. First, the times for injection of various solutions onto the capillary were partly based on theoretical calculations. Second, the set-up for capillary coating was relatively crude - the pressure gauges were prone to surges and there were leaks in the Teflon gaskets. This made controlling the length of various plugs very difficult. In

Figure 4.31 Fits to standard data used in the calculation of the activity of alkaline phosphatase molecules in the bulk assay.

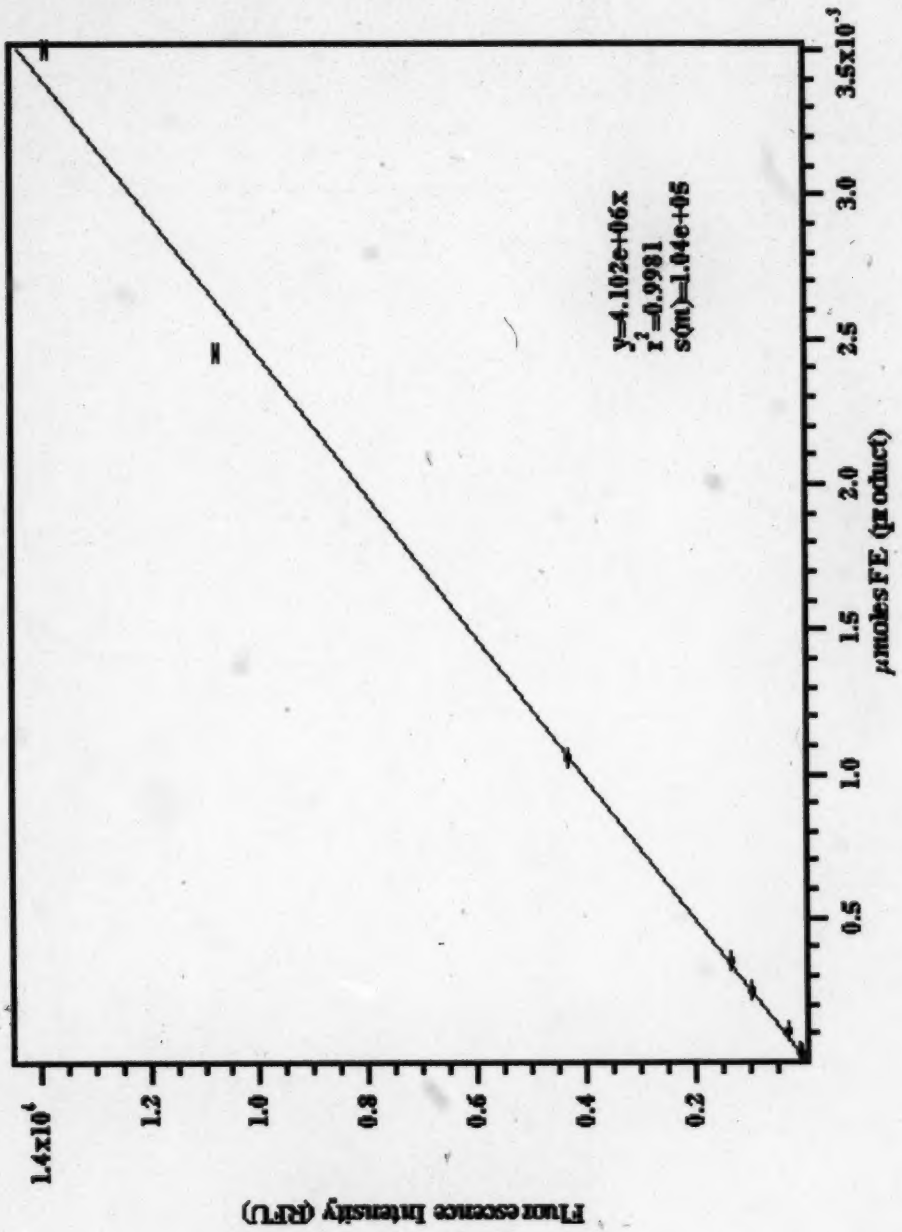


Figure 4.32 Results of the bulk (microtiter plate) assay of solubilized calf intestinal alkaline phosphatase activity. Michaelis-Menten fits to the data are shown over the [AttoPhos™] ranges specified.

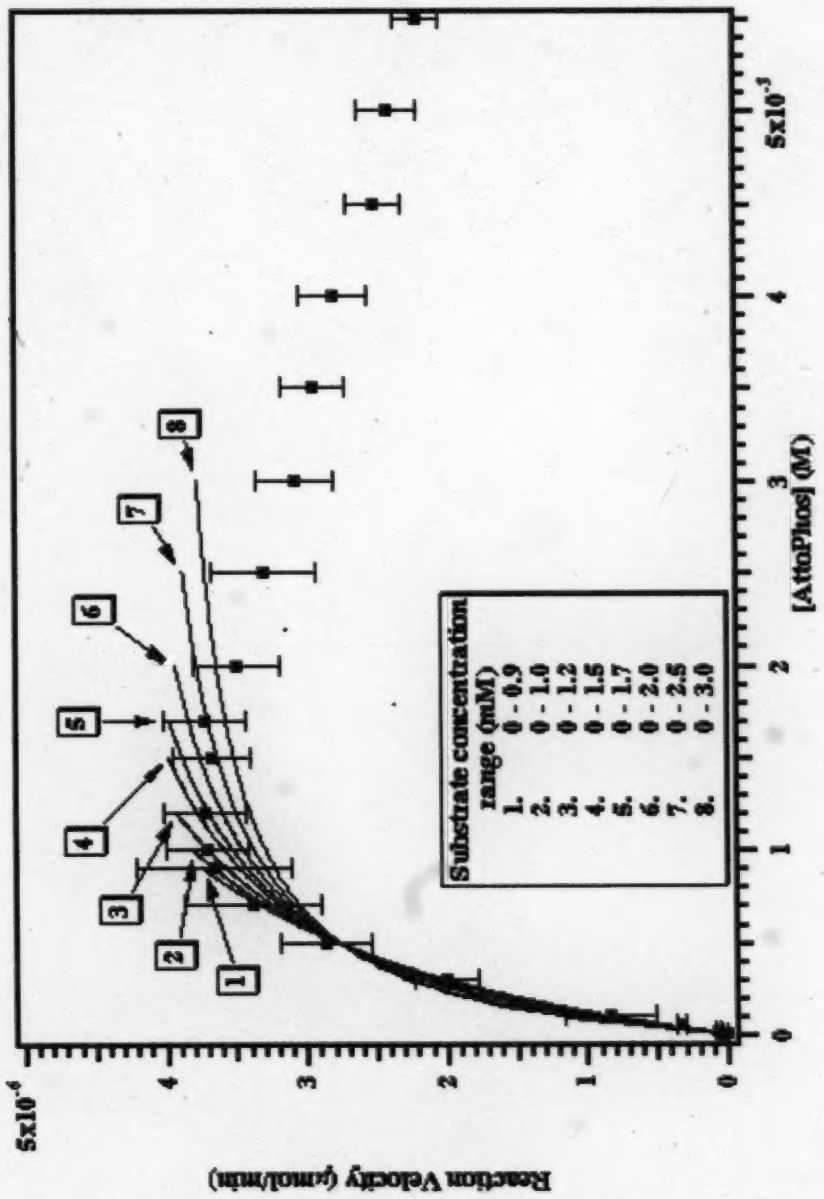


Table 4.6 a) Concentration of alkaline phosphatase obtained using BioRad Protein Stain and Molecular Devices V_{max} Kinetic Microplate Reader; **b)** Values of V_{max} and K_m for the bulk assay of activity of calf intestinal alkaline phosphatase. All values are presented with one figure beyond significant, shown in offset case.

a)

Sample	1	2	3	4	5	6	7	8	9	10	11
[AP] (mg/ml)	13.3	14.5	14.1	13.4	13.1	13.7	13.5	13.3	13.9	13.6	13.6
Average	$13.6_5 \pm 0.4_0$ mg/ml										

b)

Substrate concen. range (mM)	V_{max} (μmol/min.)		K_m (mM)
	total activity ($\times 10^{-6}$)	per molecule ($\times 10^{-14}$)	
0.001 - 0.90	$6.4_9 \pm 0.3_3$	$2.2_2 \pm 0.3_3$	$0.66_0 \pm 0.06_5$
0.001 - 1.00	$6.0_9 \pm 0.2_9$	$2.0_8 \pm 0.3_3$	$0.59_3 \pm 0.06_1$
0.001 - 1.20	$5.6_0 \pm 0.2_9$	$1.9_1 \pm 0.3_3$	$0.50_8 \pm 0.06_5$
0.001 - 1.00	$5.1_1 \pm 0.2_9$	$1.7_4 \pm 0.3_3$	$0.41_9 \pm 0.06_8$
0.001 - 1.70	$4.8_7 \pm 0.2_5$	$1.6_6 \pm 0.3_3$	$0.33_7 \pm 0.06_2$
0.001 - 2.00	$4.5_9 \pm 0.2_4$	$1.5_7 \pm 0.3_3$	$0.32_5 \pm 0.06_3$
0.001 - 2.50	$4.3_2 \pm 0.2_4$	$1.4_4 \pm 0.3_3$	$0.27_6 \pm 0.06_3$
0.001 - 3.00	$4.0_8 \pm 0.2_3$	$1.3_9 \pm 0.3_3$	$0.23_5 \pm 0.06_4$

one case, not presented here, the enzyme seemed to be immobilized prior to the heater unit. The greatest problem in the immobilization of enzyme was the irreproducibility in the number of immobilized molecules. Some capillaries, such as CI-13, had a significant number of molecules bound to the wall, while others had only a few, or even just a single molecule immobilized. Immobilizing a large number of molecules inevitably leads to many of them being too close to achieve full separation of the product peaks. However, as can be seen with capillary no. CI-18, having just a few molecules still does not guarantee full resolution. Nevertheless, the peaks could still be fitted quite well using PeakFit, by minimizing the squares of their residuals.

Most of the capillaries that were used for enzyme immobilization did not last long enough to obtain enough data for a complete Michaelis-Menten study of single enzyme molecule kinetics. Most often the capillaries would break when subjected to high voltage; sometimes they were plugged. The most likely reason in both cases is incomplete rinsing and/or particulates in any of the solutions used in immobilization. This becomes especially relevant when modifying small diameter capillaries. In fact, this was the first time that 10 μm i.d. capillaries were successfully modified in this laboratory. On the other hand, the capillaries used in the experiments which are discussed in this work lasted for a considerable period of time.

The stability of the immobilized enzyme molecules proved to be quite remarkable. All the capillaries used in assays broke before any of the enzyme molecules under study lost their activities. For the longest-lived capillary, the CI-18, the enzyme molecules survived for 9 days without an apparent loss in activity. The capillary had to be discarded because of a break, not because of enzyme's loss of activity. Undoubtedly, the enzyme's survival was aided by overnight and over-the weekend storage at 4°C. Efficient heat dissipation during electrophoresis, achieved by the use of low i.d. capillaries and heating/cooling unit, might also have played a role. Fortunately, the enzyme did not require additional metal cofactors to be present in the

electrophoresis buffer - the affinities of Zn^{2+} and Mg^{2+} for the enzyme are high[41]. Both metals are troublesome in CE in bare silica capillaries - their adsorption onto the walls causes current fluctuations and migration time irreproducibilities.

The immobilized molecules showed a wide range of V_{max} values, as might have been expected for calf intestinal alkaline phosphatase which was previously shown to be quite heterogeneous with respect to activity[42-44]. The V_{max} values ranged from a low of 2.5×10^{-14} µmol/min. for capillary no. CI-13 to a high of 74×10^{-14} µmol/min. for capillary no. CI-18. This represents a nearly 30-fold difference in V_{max} values - relatively larger than the 10-fold difference observed previously. There is however a significant difference between the activities of molecules immobilized on capillary no. CI-13 and those immobilized on capillary no. CI-18. Looking at these capillaries separately, the differences between the least and most active molecules within each capillary are reduced to about 3-fold for capillary no. CI-13 and about 11-fold for capillary no. CI-18. The smaller difference observed for capillary no CI-13 is likely due to a certain bias in choosing the peaks that were to be analyzed. Only 7 peaks were chosen for analysis and these were clearly not the most active, nor the least active, molecules in that capillary. The emphasis of this analysis was to pick fully resolved peaks. In contrast, all the molecules immobilized on capillary no. CI-18 were included in the analysis, despite their resolution. As a result, the average V_{max} value for molecules in capillary no. CI-13 is about 1/5th of that for molecules in capillary no. CI-18.

The K_m values also varied between the two capillaries and within each of the capillaries, although this variation was not as great as in the case of V_{max}. The difference between the highest and the lowest K_m value was 15-fold between the two capillaries. Within each capillary, this difference was about 4-fold for capillary no. CI-13 and about 7-fold for capillary no. CI-18. Once again, the Michaelis constant was on average larger for the second capillary.

The values for V_{max} and K_m obtained for the bulk assay were measured over a range of substrate concentrations, due to enzyme inhibition which becomes apparent at substrate concentrations above 1.0 mM. The most likely reason for the decrease in enzyme activity at these concentrations is inhibition of enzyme by orthophosphate[45,46], one of the products of the enzymatic reaction. This inhibition does not occur in capillaries where the amount of substrate, and therefore also of the product, present is very low[47]. It becomes an issue in a bulk assay, were much more substrate is available for the reaction with enzyme, which is also present in larger quantity. A reduction in orthophosphate inhibition for an immobilized alkaline phosphatase has previously been reported by Shan et al.[32], however no explanation was given for this behaviour. If the substrate concentration range of 0 - 1.0 or 1.2 mM is employed in the Michaelis-Menten analysis, V_{max} and K_m values of about $(2.0 \pm 0.1) \times 10^{-14}$ µmol/min. and 0.55 ± 0.04 mM, respectively, are obtained.

Comparing the data obtained for the immobilized enzyme molecules (**Tables 4.4** and **4.5**) to that obtained for the solubilized molecules (**Table 4.6**), there are two trends to be observed. First, the V_{max} values are significantly lower for the bulk assay as compared with either of the averaged values for the bound enzyme capillaries (average values are obtained by fitting plots of substrate concentrations vs. activities averaged over the number of peaks measured). This difference is only 3-4 fold for capillary no. CI-13 but it grows to 17-18 fold for capillary no. CI-18. Second, the K_m values are also lower for the bulk assay. The differences between the values are negligible for capillary no. CI-13 but on the order of 2-fold higher for capillary no. CI-18.

An increase in K_m value for an immobilized enzyme has been postulated by numerous researchers in the field[8,32,48]. According to Goldstein[19] the Michaelis constant for an immobilized enzyme is related to that for solubilized enzyme through the following equation:

$$K_m' = K_m \exp(ze\psi / kT) \qquad \textbf{(Equation 4.3)}$$

where K_m' is the Michaelis constant for immobilized enzyme (also known as the apparent K_m), ze is the charge on the substrate, ψ is the charge on the supported enzyme, k is the Boltzmann constant, and T is the absolute temperature. According to this equation, for the case of substrate and supported enzyme having similar charges (of the same sign), $K_m' > K_m$. This is the case for alkaline phosphatase covalently bound to modified fused silica wall acting on Fluorescent Emitter as a substrate - both have a net negative charge. This behaviour is a result of partitioning of the substrate between the enzyme support and the bulk solution phase. The effective concentration of substrate around the immobilized enzyme will be less than that for the bulk solution because of charge repulsion. The very small magnitude of this difference in this study may be due to the fact that the net charge of the supported enzyme is rather spread out because of the electrically neutral linker arms between the enzyme and the silica surface.

The situation with the V_{max} for the immobilized enzyme is a little different. According to some previous work[48], one would expect a slight decrease in the V_{max} value for an immobilized enzyme. Again, this effect is thought to be due to diffusional limitations exerted by the support, partitioning effects, enzyme modification due to immobilization, inhibitor effects, or a combination of any of these factors[49]. For single molecules of alkaline phosphatase immobilized on modified silica surface however, there is an apparent increase in this constant as compared to free enzyme in solution. This is an unexpected increase and in fact may be rather misleading for a number of reasons. First, the calculated specific activity of alkaline phosphatase in bulk solution depends on the concentration of active enzyme. At the same time, mass quantitation of protein in the bulk solution by the Bradford method does not distinguish between active and inactive enzyme, nor even between an enzyme and any other type of protein. Therefore, the calculated concentration of enzyme may be artificially high, due to partial inactivation of the enzyme or to protein additives (such as BSA) commonly

used to stabilize enzyme preparations, thus leading to a lowering of the calculated activity and hence of the V_{max} constant. Second, the Bradford method for protein quantitation can give inflated or deflated values for the mass concentration of protein depending on the standard used. In a previous quantitation of calf intestinal alkaline phosphatase purchased from Sigma, where the mass concentration was provided by the manufacturer, the Bradford method gave a mass concentration 2.3 times higher than expected when using IgG as a standard. Once again, this would obviously result in a lowered specific activity. On the other hand, on-capillary single molecule assays ensure that only active molecules of the enzyme are counted and used in the calculations of activity.

Another possibility is a much stronger degree of product inhibition than assumed in the calculations. It is possible that the inhibition occurs earlier than at the substrate concentration of 1.0 - 1.2 mM, leading to a much-lowered calculated V_{max}. The effect on the K_m would be much smaller here, since the initial portion of the curve rises rather sharply, so that even a relatively large rise in V_{max} would result in a relatively small increase of K_m. Unfortunately, efforts to lower the concentration of enzyme in the assay, which would result in lowered concentration of product, were unsuccessful; the instrument's sensitivity was inadequate.

The final possibility, although one for which there is very little precedence[50], and no easy explanation, is activation of the enzyme attached to the support. This is a rather unlikely explanation.

4.4 CONCLUSIONS

Immobilization of active enzyme molecules is a necessary step in the study of single molecule enzyme kinetics. Many immobilization methods exist now and the choice of any one of them will be dictated by the researcher's interests, experimental set-up, ability, enzyme under study, etc. Covalent attachment is definitely one of the

most widely used methods. The covalent attachment of single molecules of calf intestinal alkaline phosphatase to derivatized fused silica capillary surface was the method of choice for these experiments. The immobilized enzyme molecules exhibited high stability in the capillary reaction vessels. The immobilization procedure was relatively gentle on the enzyme, requiring no harsh chemical treatment of the enzyme.

The results of the kinetic analysis of single molecules of calf intestinal alkaline phosphatase required careful interpretation. The Michaelis constant was shown to be higher for the immobilized enzyme as compared with analogous free enzyme in solution. This could be explained by diffusional hindrances exherted on the substrate by the support, and by partitioning of the substrate between the bulk solution and the support. This behaviour was expected from previous studies.

Maximum reaction velocity was, surprisingly, higher for the immobilized enzyme than for freely solubilized counterpart. Although some degree of activation upon immobilization is possible, it is very unlikely and difficult to explain. Much simpler explanations include the following two possibilities. First, there may be large error associated with active enzyme estimation in bulk solution. This would lead to an apparent lowering of the specific activity of free solution enzyme. Second, product inhibition was observed in bulk sample. The substrate concentration at which this inhibition takes place was estimated, yet there remains a strong possibility that it might have been underestimated. This would lead to a lowered value for V_{max} in bulk solution.

Enzyme immobilization by any of the possible methods is a necessity in kinetic measurements of single molecules of enzymes. It is also the only way to approximate cellular conditions, native to many enzymes, for studies with purified, commercially available products. Because of the changes to the enzyme's environment, and possibly the enzyme itself, the results of studies on immobilized enzymes must be carefully analyzed and compared to those obtained traditionally.

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5

Conclusions and Future Work

5.1 CONCLUSIONS

It is sometimes hard to imagine that single molecule enzymology has been around for 40 years now. Its beginnings were humble - B. Rotman needed a full day (10-15 hrs) of incubation to detect, by fluorescence measurements, the presence of single enzyme molecules in suspended buffer droplets[1]. The improvements in detection techniques achieved since then have allowed for the experimental methods to become ever more complex and for the scope of the studies to broaden. Various researchers in the field now look at all sorts of aspects of single molecules in determining their static and dynamic disorders. Characteristics such as kinetic behaviour, conformational dynamics, biomechanics, reaction intermediates, diffusion, thermal denaturation, activation energy are all studied on a single molecule basis. Extending the studies from observation to active participation, force microscopy techniques allow for manipulation of single molecules. For the observation of single molecules, fluorescence is still the method of choice in most laboratories. Manipulation of single molecules is achieved with the help of atomic force microscopy and optical tweezers.

The history of single enzyme molecule work in this lab goes back about 20 years, to the time when N. J. Dovichi was still working with R. A. Keller at Los Alamos. Since then a number of papers have been published mostly dealing with alkaline phosphatase, but also looking at the original "Single Molecule" - β-Galactosidase. The discovery by Craig *et. al.*[2] of large static disorder in the activities of single molecules of calf intestinal alkaline phosphatase agreed with work done by others around the same time on different enzymes. A number of possibilities for this heterogeneity were explored to finally postulate the primary structure differences, namely post-translational modifications, as the reason behind it. More evidence was needed however, to make a clear-cut case.

I arrived in N. J. Dovichi's lab shortly after the above mentioned work was published specifically with the single molecule work in mind. Taking over after D. Craig, I was looking to answer the question of enzyme structure - activity relationship. Continuing

the work with calf intestinal alkaline phosphatase seemed to be the most logical option at that time. The enzyme was already shown to be heterogeneous and a reason for it was postulated. It was also commercially available and easily assayed by the method developed by D. Craig. Some improvements to this technique seemed possible, perhaps even necessary. The preliminary work therefore, proceeded along this line: improve the assay conditions to eliminate any influences which may differentially affect individual molecules of the sample and to improve the sensitivity of the assay; purify the enzyme into homogeneous fractions; look at these fractions and tie the heterogeneity of the enzyme to differences in structures of individual isozymes.

Unfortunately not everything went as planned in the course of my research. Improving reaction conditions over those employed previously proved to be too difficult and time consuming - essentially, the experiment was designed very well to start with. Purifying the calf intestinal enzyme further was also very difficult - its great structural complexity directly translated into great difficulty in isolating the isoforms. Since a clear link could not be drawn between the isoforms of CI-AP and the hetereogeneity in the activities of individual molecules of this enzyme, a simpler and easier model, the *E. coli* alkaline phosphatase, was chosen. This was still a structurally heterogeneous enzyme, but the differences between its isoforms were known and their purification was possible. The results obtained with that isozyme validated the change in models. The broad question of enzyme structure-activity relationship was answered for that simple model and only a more specific question of carbohydrate content's influence on activity remains. To answer this question another alkaline phosphatase needs to be found. Among four other alkaline phosphatases studied briefly, the kidney isoforms of cow or pig appear to be good choices for the future.

Successful studies on single molecules of *E. coli* alkaline phosphatase were possible only after some improvements to the set-up used previously for single molecule studies on calf intestinal alkaline phosphatase. The less active *E. coli* isozyme needed

much longer incubation times. This made the experiments very long and reduced the amount of data that could be generated. To change that the enzyme had to be assayed under conditions closer to its optimal. Attempt at changing the buffer to one that would stimulate its activity and provide a pH closer to its optimum failed. As with the previous attempts with calf intestinal isozyme, the preferable assay conditions were not the preferable CE conditions. Where the pH couldn't be used to help, temperature turned out to be the answer. An addition of the Peltier-driven temperature controller allowed for the shortening of incubation times by half, while at the same time having the benefit of keeping constant day-to-day temperature, an important factor for an enzyme whose activity increases by 5% with each °C. With the assay sensitivity thus improved the task of looking at differences in individual molecules became easier. Purification of the *E. coli* alkaline phosphatase isoforms was the next necessary step. Isoelectric focusing in slab gels was able to resolve the individual species, and the use of non-denaturing visualization procedure allowed for the recovery of active enzyme. Thus prepared, it was now possible to study the activity of individual molecules in light of their structure. The heterogeneity of this enzyme's commercial preparations proved to be the result of not the differences in the enzyme's individual isoforms' structures, but rather the result of the degradation of the enzyme in those preparations. Further evidence for this degradation was provided by gel electrophoresis of the commercial and purified samples. When the isoforms were purified to high homogeneity, the differences all but disappeared, to within the experimental error, for the molecules of the same isoform. The differences between molecules of the different isoforms also became very small. This is not surprising when the structures of the three isoforms are taken into consideration. The minute differences between the three "native" isoforms do not warrant the large differences previously observed in the activities of the molecules in bulk samples. Nevertheless, it would be rash to assume that the isoforms must be identical in their reaction rates. After all, their structures, although very similar, are not identical. The evidence shows, that there very well may be small differences in

activities of the isoforms - these differences, however are not nearly as large as previously seen.

In another study of individual molecules of *E. coli* alkaline phosphatase thermal denaturation of this enzyme was shown to be a catastrophic process. This was previously shown to be the case for the calf intestinal isozyme as well. Exponential decrease in the number of surviving molecules with increasing heating time was observed. This decrease was not paralleled by an identical decrease in the activities of survivors as would have to be the case if thermal denaturation obeyed the Cheshire Cat model. The conclusion as to the mode of death was drawn carefully, because the experiment had a possible flaw - some of the survivors could in fact be molecules that were denatured but were able to re-fold into their active shapes in the course of the incubation. In the end, the evidence still pointed towards a catastrophic death as opposed to a gradual unfolding of the enzyme with associated gradual decreases in activities of individual molecules (the Cheshire Cat model). In addition, activation of the enzyme upon very short heating at high temperature has been observed, in accordance with the high thermal stability of this particular alkaline phosphatase and some previous studies showing increasing reaction rates with increasing temperatures, all the way to 80°C[3].

The single molecule studies on this enzyme helped to show that the there is a strong correlation between the enzyme's primary structure and its function (activity). This correlation appears to be stronger than that proposed by Yeung *et al.*[4], who suggested that activity differences between various isoforms are due to secondary structure fluctuations.

In the final part of the research project single molecule kinetics were studied. Immobilization of active enzyme molecules is a necessary step in the study of single molecule enzyme kinetics. Many immobilization methods exist now and the choice of any one of them will be dictated by the researcher's interests, experimental set-up, ability, enzyme under study, etc. Covalent attachment is definitely one of the most widely used

methods. The covalent attachment of single molecules of calf intestinal alkaline phosphatase to derivatized fused silica capillary surface was the method of choice for these experiments. The immobilized enzyme molecules exhibited high stability in the capillary reaction vessels. The immobilization procedure was relatively gentle on the enzyme, requiring no harsh chemical treatment of the enzyme.

The results of the kinetic analysis of single molecules of calf intestinal alkaline phosphatase required careful interpretation. The Michaelis constant was shown to be higher for the immobilized enzyme as compared with analogous free enzyme in solution. This could be explained by diffusional hindrances exerted on the substrate by the support, and by partitioning of the substrate between the bulk solution and the support. This behaviour was expected from previous studies.

Maximum reaction velocity was, surprisingly, higher for the immobilized enzyme than for freely solubilized counterpart. Although some degree of activation upon immobilization is possible, it is very unlikely and difficult to explain. A much simple explanation are the following two possibilities. First, there may be large error associated with active enzyme estimation in bulk solution. This would lead to an apparent lowering of the specific activity of free solution enzyme. Second, product inhibition was observed in bulk sample. The substrate concentration at which this inhibition takes place was estimated, yet there remains a strong possibility that it might have been underestimated. This would lead to a lowered value for V_{max} in bulk solution.

Enzyme immobilization by any of the possible methods is a necessity in kinetic measurements of single molecules of enzymes. It is also the only way to approximate cellular conditions, native to many enzymes, in studies with purified, commercially available products. Because of the changes to the enzyme's environment, and possibly the enzyme itself, the results of studies on immobilized enzymes must be carefully analyzed and compared to those obtained traditionally.

Single molecule enzymology is to classical enzymology as a one-on-one encounter is to sociology. Instead of looking at an ensemble average and from there inferring information about individual molecules, one is able to look directly at them. Not surprisingly individual molecules are just that - individual. They differ from each other as we differ amongst ourselves. When they are identical and present in identical environments, they seem to behave exactly the same, not unlike single-egg twins. As the differences in their structures become more pronounced, their behaviour starts to become more and more varied. It is a very rich world that surrounds us and the only way to do it justice is to look at it in as close a detail as possible. Averaging is simply stereotyping.

5.2 FUTURE WORK

If one had to follow up on every single idea that comes to mind as the research project is being carried out, a thesis would never be finished. Not only are some ideas worth more than others, they all inevitably lead to more ideas. So for all those side roads, we have the future work section - and there is always plenty of it. Especially in a field as potentially diverse as single molecules.

More work remains to be done on the question of glycosylation vs. activity. Since there are so many glycoproteins around, it would be great to be able to pin-point the influence that the long carbohydrate chains have on the behaviour of individual enzyme molecules. As previously suggested, bovine or porcine kidney alkaline phosphatases, both commercially available, seem to be good choices to follow up on the calf intestinal enzyme study. Future work with these molecules would necessarily involve further purification of the isozymes. The attempts so far were not sufficiently successful. Perhaps more work is required in the isoelectric focusing of these enzymes. Perhaps it would be easier to deglycosylate them fully, while leaving their activities intact, to see if that brings about the convergence in their activity distributions. Finally, it may be better to find yet another model. In an ideal situation, an enzyme could be synthesized *in-vitro*, active but devoid of

sugars. Its counterpart would have to be synthesized alongside, this time with the inclusion of sugars. The two could then be studied by our proven methods in order to finally fully answer the question of structure - activity relationship.

Some more work could even be done on the *E. coli* model. Since it is extremely difficult to see the differences in the activities of the individual isoforms, due to their minute structural differences, amplifying these differences would help to follow their influence on the activities of isoforms. Such amplification could be achieved by partial and controlled proteolytic digestion of the polypetide chain. Seeing the activities change as the polypeptide chain gets shorter and the enzyme begins to unfold would help us understand the broader issue of structure-function relationship. In the same vein, thermal denaturation study could be followed by single molecule studies of other denaturation methods, such as acid/alkaline denaturation, chelator denaturation, or denaturation due to the presence of known denaturants such as DTT or β-mercaptoethanol.

To help with any such studies, the sensitivity of the instrument and of the assay method should be continually improved. The greater the sensitivity, the more accurate are the conclusions from the experiments. Adjusting temperature has already been shown to help. Adjusting pH, buffer ionic strength, and additive content, changing the substrate or the buffer type are all potentially beneficial. Lowering background in fluorescence measurements, using smaller id capillaries, improving collection efficiency and signal amplification could also help. Not all of it can be successfully applied - some changes are not compatible with the general assay method or with the instrumental capability. Yet even small improvements can add up to new findings among the old models.

The most beneficial change however, would be, in my opinion, a switch from time averaged experiments to real-time detection and monitoring of enzyme behaviour. The sheath-flow cuvette-based instrument has served us well in our ventures into the world of single molecules. The future however, seems to lie in fluorescence microscopy, where molecules entrapped within the detection volume can be observed at will, as they perform

their functions. These methods are already in use by a number of research groups and their application brings fascinating insight into the workings of single molecules. So much more could be discovered, even about our beloved alkaline phosphatase. Since its complete reaction mechanism is still not known, real-time studies of its kinetics could go a long way towards the elucidation of the full mechanism, and by extension, of many unknown mechanism.

The single molecule field is growing and is poised to grow for years to come. Now, that we have acknowledged the individuality of chemical molecules, we are bound to seek ever greater knowledge about them.

5.3 BIBLIOGRAPHY

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